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The Roles of Nuclear Receptor NR4A1 in Cancer Cell Proliferation and Skeletal Muscle Differentiation

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The Roles of Nuclear Receptor NR4A1 in Cancer Cell Proliferation and Skeletal Muscle Differentiation

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DEDICATION

This dissertation is dedicated to my husband and our families for their love and support during my time in graduate school.

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ABSTRACT

Nuclear receptors (NRs) constitute a major class of drug targets in the treatment of various cancer types. NRs respond to cellular signals and become activated upon ligand binding to transcriptionally modulate expression of target genes. NR4A1 (Nur77) is a member of the NR4A family of nuclear receptors and displays an oncogenic profile in many cancer models. It is often upregulated in adult solid malignancies and is known to promote cell proliferation and survival. Knockdown studies of *NR4A1* in cancer cell lines result in decreased cell growth and angiogenesis and increased apoptosis, suggesting NR4A1 is an oncogenic protein. Due to the elevated levels of NR4A1 in cancer, it is important to determine the regulatory mechanisms behind this expression pattern.

One such mechanism is through microRNAs (miRNAs), which regulate gene expression by binding to the 3′UTR of target mRNA and effectively inhibit translation into protein. Prior to this study, no miRNAs had been identified to directly target *NR4A1*. By using luciferase reporter assays, we identified miR-124, miR-15a, and miR-224 as potential *NR4A1* regulators. The direct binding of these miRNAs to their potential seed regions within the 3′UTR of *NR4A1* was confirmed by mutagenesis of their respective seed sequences. This abrogated the binding and thereby confirmed the direct targeting of these miRNAs to these particular sequences. To further study the relationship between NR4A1 and these miRNAs, we analyzed endogenous expression levels in several pediatric cancer cell lines. *NR4A1* was upregulated in RD, Rh41, and Rh30 rhabdomyosarcoma cells and D341 and Daoy medulloblastoma cells as well as NB3 neuroblastoma cells. All three miRNAs were downregulated in Daoy cells. Considering that miR-124 is highly expressed in the brain and is a tumor suppressor, we decided to investigate the functional significance between NR4A1 and miR-124 in Daoy cells. We found that miR-124 could decrease NR4A1 mRNA and protein levels as well as the expression of several NR4A1 target genes. Overexpression of NR4A1 led to enhanced cell viability and proliferation while knockdown resulted in the opposite phenotype. Furthermore, stable expression of miR-124 in Daoy cells resulted in decreased proliferation and smaller spheroid formation. Lastly, we examined expression levels in granule neuron precursors (GNPs), which are the most common cell type in the cerebellum where medulloblastoma arises. Interestingly, there was an inverse expression pattern in which miR-124 was increased while *Nr4a1* was decreased in the differentiated GNPs, suggesting a potential role for NR4A1 in neuronal development.

In addition to cancer cell proliferation, the role of NR4A1 in skeletal muscle differentiation was also explored. We found that *NR4A1* increased during the differentiation of human LHCN myoblasts, and that knockdown of *NR4A1* impairs differentiation and reduces expression of myogenic markers in LHCN as well as SkMC, and HSMM primary human skeletal muscle cells. This data agrees with previous studies performed in mouse models and mouse C2C12 cells showing increased *Nr4a1* expression during differentiation as well as the ability of NR4A1 to enhance muscle mass and myofiber size.

Together, these two studies highlight two different and opposing functional roles of NR4A1 in medulloblastoma and skeletal muscle. The first study identified three miRNAs capable of directly targeting and suppressing *NR4A1* and also provides a rationale for the use of miRNA mimics as a potential therapeutic in cancers with high NR4A1 expression. In the second study, we provided evidence that further confirms the pro-myogenic function of NR4A1 during skeletal muscle differentiation. It is important to understand this basic biology as it can help further understand and treat diseases related to muscle such as rhabdomyosarcoma and muscular dystrophy.

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CHAPTER 1. INTRODUCTION

Nuclear Receptors

Discovery and classification

Before the word 'hormone' was coined in 1905 by Ernest Starling or the word 'receptor' by Paul Erhlich in 1907, not much was known about hormones or nuclear (hormone) receptors [1]. The first NRs cloned were the glucocorticoid and estrogen receptors in 1985 and 1986, respectively [2, 3], although the estrogen receptor was originally isolated in 1958 by Elwood Jensen [4]. Preceding the discovery of these receptors, cortisone, a steroid that binds the glucocorticoid receptor, and thyroxine, an iodoamino acid that binds the thyroid receptor, were the first ligands to be isolated and analyzed for their structures in 1926 [5, 6]. Soon after in the 1920s and 1930s, the pancreatic hormone insulin was characterized along with the steroid hormones estrogen, testosterone, and progesterone [1]. It is now known that humans have a total of 48 nuclear receptors termed the nuclear receptor superfamily, as listed in **Table 1-1** [7].

Over the years as more and more NRs were discovered, there was much confusion as to the nomenclature of these genes. Hence, in 1999 a group of scientists came together to formally name these receptors based on their phylogeny [8]. These 48 nuclear receptors have since been classified into 6 subfamilies, with the first subfamily, the thyroid hormone receptor-like family, having 8 subgroups. These groupings are based on sequence homology [9-11]. There are also several ways of referring to each receptor. Other than spelling out the full name of the receptor, it can also be identified by its nuclear receptor nomenclature committee (NRNC) symbol, its abbreviation, or its gene name. For example, nerve growth factor-induced clone B, a member of the nerve growth factor IB-like subgroup, is part of the 4th subgroup and is the first member of its family and therefore has the NRNC symbol NR4A1. Its abbreviation is Nur77, although it has many other common names including NGFIB, NR4A1, and TR3, and lastly its gene symbol is *NR4A1*.

They can also be grouped into liganded NRs, adopted NRs, and orphan NRs. Liganded NRs are those which have a known ligand, such as the estrogen receptor with estrogen acting as its ligand. On the other hand, orphan receptors have no known endogenous ligand, such as COUP-TF. And as the name implies, adopted receptors are those which were once orphans but now have an identified ligand, such as FXR found to be activated by bile acids [12]. The initial discovery of nuclear receptors was based on using ligands to identify the receptor they bind to, but as technology improved it became possible to discover receptors without knowing their ligands, hence the appearance of adopted nuclear receptors.

Table 1-1. Nuclear receptor superfamily.

Subfamily	Group	Name	Abbreviation	Ligand	Disease implicated	Therapeutic drug name
Thyroid hormone receptor-like	Thyroid hormone receptor	thyroid hormone receptor α	TRα	thyroid hormone	thyroid resistance syndrome, thyroid cancer	evothyroxine, levothyroxine, liothyronine
		thyroid hormone receptor β	TRβ	thyroid hormone	hypercholesterolemia	
	Retinoic acid receptor	Retinoic acid receptor α	RARα	retinoic acids	acute promyelocytic leukemia, kidney disease, Alzheimer's disease, skin diseases, cancers	all-trans retinoic acid (ATRA)
		Retinoic acid receptor β	RARβ	retinoic acids		
		Retinoic acid receptor γ	RARγ	retinoic acids		
	Peroxisome proliferator-activated receptor	Peroxisome proliferator-activated receptor α	PPARα	Fatty acids and prostaglandins	Type II diabetes, atherosclerosis, obesity, hyperlipidemia	fibrates (clofibrate, gemfibrozil, fenofibrate)
		Peroxisome proliferator-activated receptor β/δ	PPARβ/δ	Fatty acids and prostaglandins	Type II diabetes, atherosclerosis, obesity, hyperlipidemia	
		Peroxisome proliferator-activated receptor γ	PPARγ	Fatty acids and prostaglandins	Type II diabetes, atherosclerosis, obesity, hyperlipidemia, anaplastic cancer	thiazolidinediones (roziglitazone, pioglitazone, perflurooctanoic acid), RS544
	Rev-ErbA	Reverse-Erb α	REV-ERBα	heme		
		Reverse-Erb β	REV-ERBβ	heme		
	RAR-related orphan receptor	RAR-related orphan receptor α	RORα	cholesterol and ATRA	atherosclerosis	
		RAR-related orphan receptor β	RORβ	cholesterol and ATRA		
		RAR-related orphan receptor γ	RORγ	cholesterol and ATRA		

Table 1-1. Continued.

Subfamily	Group	Name	Abbreviation	Ligand	Disease implicated	Therapeutic drug name
	Liver X receptor-like	Liver X receptor α	LXRα	oxysterols	Alzheimer's disease, atherosclerosis, dyslipidemia, non-alcoholic fatty liver disease, breast cancer	TO901317, GW3965, N-Acylthiadiazolines
		Liver X receptor β	LXRβ	oxysterols	atherosclerosis, dyslipidemia	
		Farnesoid X receptor α	FXRα	oxysterols, bile acids	cholestasis, hypercholesterolemia, biliary cirrhosis, non-alcoholic fatty liver disease	guggulsterone, chenodeoxycholic acid, fexaramine
		Farnesoid X receptor β	FXRβ	oxysterols		
	Vitamin D receptor-like	Vitamin D receptor	VDR	vitamin D and lithocholic acid	hypocalcemia, osteoporosis, renal failure, colon cancer, diabetic nephropathy, hypertension, atherosclerosis	calcitriol, doxercalciferol
		Pregnane X receptor	PXR	xenobiotics and endobiotics	cholestatic liver disease, hyperbilirubinemia, liver injury, cancer	rifampicin
		Constitutive androstane receptor	CAR	xenobiotics, androstane	cholestatic liver disease, type II diabetes	phenobarbitol
Retinoid X Receptor-like	Hepatocyte nuclear factor-4	Hepatocyte nuclear factor-4 α	HNF4α	fatty acids, palmitic acid	diabetes	
		Hepatocyte nuclear factor-4 γ	HNF4γ	fatty acids		
	Retinoid X receptor	Retinoid X receptor α	RXRα	9-cis retinoic acid and docosahexanoic acid	metabolic diseases, cancers (skin cancer, cutaneous T cell lymphoma)	bexarotene

Table 1-1. Continued.

Subfamily	Group	Name	Abbreviation	Ligand	Disease implicated	Therapeutic drug name
		Retinoid X receptor β	RXRβ	9-cis retinoic acid and docosahexanoic acid		
		Retinoid X receptor γ	RXRγ	9-cis retinoic acid and docosahexanoic acid		
	Testicular receptor	Testicular receptor 2	TR2			
		Testicular receptor 4	TR4			
	TLX/PNR	Tailless homolog orphan receptor	TLX			
		Photoreceptor cell-specific nuclear receptor	PNR			
	COUP/EAR	Chicken ovalbumin upstream promoter-transcription factor I	COUP-TFI			
		Chicken ovalbumin upstream promoter-transcription factor II	COUP-TFII			
		V-erbA-related	EAR-2			
Estrogen Receptor-like	Estrogen receptor	Estrogen receptor-α	ERα	estrogens	breast cancer, ovarian cancer, prostate cancer, colon cancer, osteoporosis	tamoxifen, raloxifene, gen-estein, diethylstilbestrol, equineestrogens
		Estrogen receptor-β	ERβ	estrogens	breast cancer, ovarian cancer, prostate cancer, colon cancer, osteoporosis	
	Estrogen related receptor	Estrogen-related receptor-α	ERRα			
		Estrogen-related receptor-β	ERRβ			

Table 1-1. Continued.

Subfamily	Group	Name	Abbreviation	Ligand	Disease implicated	Therapeutic drug name
		Estrogen-related receptor-γ	ERRγ			
	3-ketosteroid receptors	Glucocorticoid receptor	GR	glucocorticoids, cortisol	inflammatory diseases (Inflammatory bowel syndrome, auto immune disorder, rheumatoid arthritis, gout, asthma, cancer)	dexamethasone, cortisol, prednisolone, RU486
		Mineralocorticoid receptor	MR	mineralocorticoids and glucocorticoids (aldosterone)	heart failure, hypertension, cardiovascular disease, kidney disease	spironolactone, eplerenone
		Progesterone receptor	PR	progesterone	breast cancer, endometriosis	RU486
		Androgen receptor	AR	androgens, testosterone	prostate cancer, osteoporosis	flutamide, bicalutamide
Nerve Growth Factor IB-like	NGFIB/NURR1/NOR1	Nerve growth factor induced gene B	NGFIB	unsaturated fatty acids		
		Nuclear receptor related 1	NURR1		Parkinson's disease	
		Neuron-derived orphan receptor 1	NOR1			
Steroidogenic Factor-like	SF1/LRH1	Steroidogenic factor 1	SF1	phosphatidylinositols		
		Liver receptor homolog-1	LRH-1	phosphatidylinositols		
Germ Cell Nuclear Factor-like	GCNF	Germ cell nuclear factor	GCNF			

Table 1-1. Continued.

Subfamily	Group	Name	Abbreviation	Ligand	Disease implicated	Therapeutic drug name
Miscellaneous	DAX/SHP	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1	DAX1			
		Small heterodimer partner	SHP		obesity	

Structure and function

Structure. By definition, nuclear receptors are also classified as transcription factors, meaning they can bind the promoters of their target genes to modulate gene expression. The typical structure of a nuclear receptor consists of a well-conserved DNA-binding domain (DBD), a moderately-conserved C-terminal ligand binding domain (LBD), and a highly variable N-terminal region containing a ligand-independent transactivation domain called Activation Function 1(AF-1) as depicted in **Figure 1-1A** [3, 10, 13]. The N-terminal region interacts with coregulatory proteins such as coactivators that enhance the transcription of NR target genes [14]. A closer look at the DBD reveals two zinc-finger domains as well as the P-box, which is a motif involved in DNA-binding specificity as well as dimerization with other nuclear receptors as homodimers or heterodimers. In addition to the promoter region, nuclear receptors may also bind enhancer and intronic regions of target genes. Nuclear receptors can bind to these regions, or more specifically their response elements, either as monomers, homodimers, or heterodimers, where heterodimers typically include binding with RXR [14]. Orphan receptors typically bind as monomers such as the NR4A family. However, there are two nuclear receptors, DAX1 and SHP, that surprisingly do not contain a DBD and mainly act as corepressors [15]. There is a flexible hinge region positioned between the DBD and LBD that contains the nuclear localization signal (NLS) responsible for facilitating the import of the receptor inside of the nucleus. The LBD is the largest domain and as its name implies, its function is dependent on the binding of small lipophilic ligands in its hydrophobic ligand binding pocket (LBP) [16]. Although the structure of the LBD is well conserved, the LBP can greatly vary in size. For example, some nuclear receptors such as SF-1 have a large LBP of about 1600 Å3, while some orphan receptors completely lack an LBP [15, 17, 18]. For instance, the orphan receptor NR4A2 contains bulky hydrophobic amino acid side chains in place of its LBP [18]. In addition to the LBP, the LBD also contains a dimerization interface and an additional transactivation domain called Activation Function 2 (AF-2), which interacts with and recruits coregulators and also contains a second NLS [19].

Function. Nuclear receptors function to regulate and facilitate a wide variety of biological processes including metabolism, reproduction, development, aging, and homeostasis [20]. Many of these processes are dependent upon ligands to activate their respective nuclear receptors, which in turn act as intermediates to confer signals to downstream events [19]. Ligands can include a plethora of signaling molecules such as hormones, fatty acids, xenobiotics, cholesterol, vitamin D, steroids, and retinoids [14, 21]. In the case of orphan receptors, which account for about half of all nuclear receptors, these can be induced by many factors such as stress, cytokines, and mitogens [14].

There are three main levels to nuclear receptor function; repression, derepression, and transcriptional activation [19]. In the absence of a ligand, nuclear receptor activity is suppressed by the binding of a corepressor complex, which typically contains histone deacetylase (HDAC) activity. These corepressor complexes commonly contain the subunits SMRT/NCoR2 or NCoR1, which directly bind the receptor [14]. Ligand binding can occur in either the cytoplasm or the nucleus, and receptors bound by ligands in the

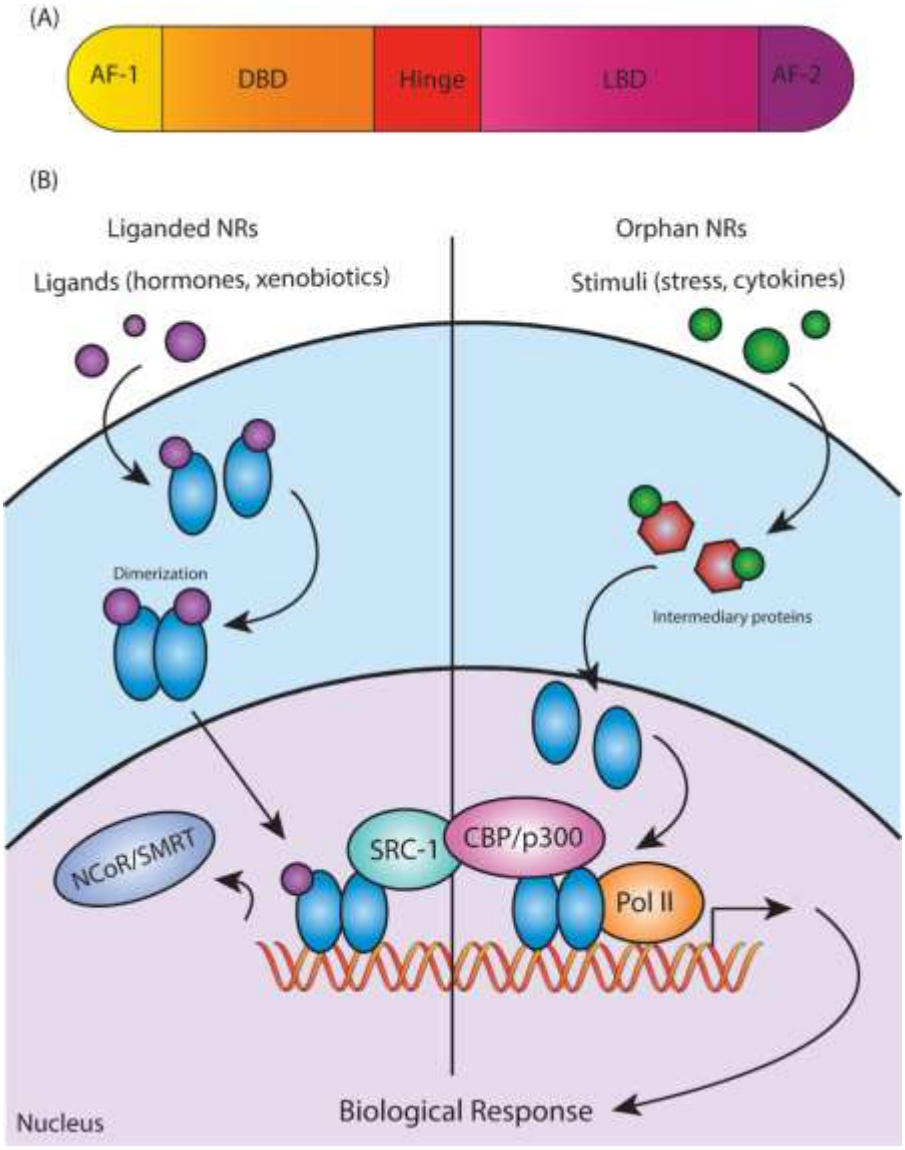

Figure 1-1. The structure and function of liganded and orphan nuclear receptors. **(A)** Typical nuclear receptors consist of a DNA-binding (DBD) and ligand-binding domain (LBD) and two transactivation function domains (AF-1 and AF-2) that recruit coregulators. **(B)** Upon activation by various stimuli, nuclear receptors will heterodimerize and bind to their target genes in conjunction with recruitment of coactivators (SRC-1 and CBP/p300) and release of corepressor (NCoR/SMRT).

cytoplasm will translocate to the nucleus [22]. Upon ligand binding, the nuclear receptor undergoes a conformational change and derepression occurs in which the corepressor complex is replaced with a coactivator complex containing histone acetyltransferase (HAT) activity. This HAT activity allows for increased transcriptional activity via chromatin decondensation. In the last stage of nuclear receptor function, the coactivator complex is replaced by another coactivator complex and the nuclear receptor is now able to bind with the promoter of its target gene to induce transcriptional activation of that gene via recruitment of transcriptional machinery such as RNA polymerase II as shown in **Figure 1-1B** [14, 15, 23]. More than 350 coactivators have been identified, including the more common ones like steroid receptor coactivator 1 (SRC-1), steroid receptor coactivator-2 (SRC-2), p300, and cAMP response element-binding protein (CBP) that are likely among the first to be recruited by nuclear receptors [14, 15, 24-26]. On the other hand, nuclear receptors may mediate repression of target gene expression by recruiting corepressors such as the nuclear receptor corepressor (NCoR), silencing mediator for retinoid and thyroid hormone receptors (SMRT), and histone deacetylase 3 (HDAC3) [27-30]. Nuclear receptors can also mediate gene repression by interacting with other transcription factors including activation protein-1 (AP-1) and nuclear factor kappa B (NF-kB) [31, 32]. Typically, unliganded nuclear receptors repress their target genes, while liganded receptors mediate activation of their target genes. Of course, this is a simplistic description of nuclear receptor function. Some nuclear receptors such as orphan receptors may not follow these steps since they may or may not be activated by a ligand [19]. Instead, they may be constitutively active such as the orphan nuclear receptor NR4A1 [33].

In addition to their transcriptional activity, nuclear receptors can also interact with other signaling pathways to impose negative or positive effects on their downstream signaling. Their activity and function can also be affected by post-translational modifications such as phosphorylation, acetylation, methylation, sumoylation, and ubiquitination [15, 34-37]. These interactions can also be dependent on the cell and tissue type.

Role in cancer

Nuclear receptors are molecular targets for approximately 13% of FDA-approved drugs [38], and inhibiting this receptor signaling has proved to be beneficial in treating cancer. Since nuclear receptors play a variety of vital roles, disruption or deregulation of their functions can lead to serious consequences. However, their ability to bind ligands makes them therapeutic targets in certain diseases and cancers. Agonists (molecules that enhance receptor function) and antagonists (molecules that disrupt or inhibit receptor function) can play important roles in drug discovery. Certain compounds with agonist/antagonist properties are classified as selective nuclear receptor modulators (SNuRMs), which act as ligands but differ in their activities from the natural endogenous ligands [15, 20]. More specifically, there are classes of compounds for certain receptors such as selective estrogen receptor modulators (SERMs) for ER, selective androgen receptor modulators for AR, and selective peroxisome proliferator-activated receptor

modulators for PPAR [39]. The more common nuclear receptors targeted in cancer therapy are the estrogen receptor (ER) in breast cancer, the androgen receptor (AR) in prostate cancer, the retinoic acid receptor (RAR) in acute promyelocytic leukemia [15], and the vitamin D receptor in breast and colon cancer.

In ER-positive breast cancer, estrogen signaling has been found to promote tumor growth and cancer progression, and treatment with SERMs is used to block this signaling. These modulators function by affecting the recruitment of certain coactivators and corepressors in a tissue-dependent manner [40-42]. For example, the SERMs tamoxifen and raloxifene increase corepressor recruitment in mammary cells, resulting in inhibition of estrogen signaling and decreased breast cancer growth. On the other hand, tamoxifen acts as an agonist in endometrial cells and mediates coactivator recruitment, leading to a higher risk of endometrial cancer [43]. However, raloxifene does not have this agonistic effect in endometrial cells. This is most likely due to preferential binding of coregulators to the AF-1 domain depending on certain factors such as tissue and cell type [44-48]. SERMs can also act as agonists in other tissues including bone, uterine, and cardiovascular tissue [49, 50]. This kind of tissue selectivity can be beneficial since estrogen signaling can be inhibited in breast cancer while still allowing for normal function in other tissues. This can also be useful in the case of osteoporosis in which raloxifene exerts protective effects while avoiding the increased risk of endometrial cancer that could result from tamoxifen treatment in menopausal women [20, 51].

Another major nuclear receptor being targeted in disease is the glucocorticoid receptor (GR) by dexamethasone and prednisolone in inflammatory diseases such as rheumatoid arthritis, immunological disorders, and cancer [20]. These synthetic ligands function as agonists of GR and enhance its ability to disrupt NF-kB and AP-1 activities, leading to decreased proinflammatory cytokines and tumor necrosis factor-α, and ultimately resulting in reduced inflammation [52-54]. However, continued treatment with dexamethasone and prednisolone can lead to serious side-effects including diabetes and osteoporosis [55].

Additional receptor targets include PPARα by fibrates (clofibrate, gemfibrozil, fenofibrate) in hyperlipidemia, PPARγ by thiazolidinediones (roziglitazone, pioglitazone, perflurooctanoic acid) in type II diabetes, RXR by bexarotene and alitretinoin in cancer, RAR by all-trans retinoic acid (ATRA) in acute promyelocytic leukemia, and AR by flutamide and bicalutamide in prostate cancer [15, 56-60]. The xenobiotic receptors CAR and PXR can also play a role in cancer by mediating the metabolism of cancer drugs and drug resistance. PXR is commonly used in the pharmaceutical industry to identify dangerous drug-drug interactions when screening for new cancer therapies [61]. Furthermore, agonists of PXR have therapeutic potential in treating cholestatic liver disease and preventing hyperbilirubinemia [22, 62]. Future drug targets include FXR in nonalcoholic steatohepatitis and cholestatic liver disease and LXR in atherosclerosis and Alzheimer's disease by using agonists of these receptors [30, 61, 63]. Many other nuclear receptors are therapeutic targets in various diseases as listed in **Table 1-1** [59, 64, 65].

NR4A Family

Discovery

The NR4A family consists of NR4A1 (Nur77, NGFI-B, TR3), NR4A2 (Nurr1), and NR4A3 (Nor-1). They were first characterized as immediate-early response genes induced by nerve growth factor in PC12 cells [66, 67]. NR4A1 was first isolated in 1989 by Chang et al. from human prostate in which they found that NR4A1 was a 64 kDA receptor protein that could bind to DNA [68]. This was following the discovery of Nr4a1 in the mouse since Chang et al. noted that human NR4A1 had 86% nucleotide and 91% amino acid sequence homology to mouse Nr4a1 [68]. Shortly thereafter, *Nr4a2* was identified and cloned from a mouse brain cDNA library by Law et al. and subsequently from fetal human tissue [69-72]. *Nr4a2* was found to be highly expressed in the brain compared to other tissues, suggesting an important role for NR4A2 in the central nervous system [69]. Lastly, *Nr4a3* was first identified in forebrain neuronal cells that were undergoing apoptosis. NR4A3 appropriately stands for neuron derived orphan receptor and was found to encode a 68 kDa protein [73].

Structure and function

Structure. As mentioned, the NR4A family has been classified as orphan receptors due to the lack of endogenous ligands. However, a recent report claims they are no longer orphans since there are both synthetic and natural compounds that have been shown to bind the LBD of all three NR4As [74]. Additionally, unsaturated fatty acids (UFAs) have been found to act as natural endogenous ligands by binding to the LBD of NR4A1 and NR4A2 [75, 76]. Interestingly, the LBDs of NR4A1 and NR4A3 contain hydrophilic surfaces as opposed to the hydrophobic LBP of liganded nuclear receptors [77, 78]. In addition, the LBDs of the NR4A family completely lack an LBP and classical coactivator binding site and instead have bulky hydrophobic side chains in place of an LBP where agonists would normally bind [18, 79]. For this reason, it was believed there could be no ligand for the NR4As, though this theory has been disproven by the discovery of small-molecule NR4A1 agonists [75]. Since NRs commonly undergo conformational changes upon ligand binding, it is possible that the NR4As reveal a binding pocket in response to ligand interactions. This was proven to be true for NR4A1 in which the UFA arachidonic acid induced a conformational change, allowing it to bind to the LBD of NR4A1 [75]. In addition, arachidonic acid was found to preferentially bind to NR4A1 oligomers as opposed to monomers and appeared to stabilize these oligomers [75].

In terms of sequence homology between the NR4A members, their DBDs and LBDs are well conserved with about 91-95% homology in the DBD and about 60% in the LBD, however the N-terminal containing the AF-1 domain is highly divergent [70, 80, 81]. This higher level of variation within the AF-1 domain likely recruits different cofactors for each NR4A in response to stimuli, which may explain the differential

binding of the NR4As to their target gene promoters, thereby resulting in the unique responses and functions of each NR4A receptor. More specifically, cofactors have been shown to bind NR4A1 protein between helices 11 and 12 in a hydrophobic region [33, 82, 83]. In terms of the NR4A1 gene structure, it is known to have seven exons, with the AF-1 domain in exon 2, the DBD in exons 3 and 4, and the LBD in exons 5 to 7 [70]. The recruitment of coregulators has been shown to involve the AF-1 domain to mediate NR4A-dependent transcription [33, 77, 78, 84, 85].

Function. Like all nuclear receptors, the NR4As function as transcription factors and bind the promoters of their target genes to modulate their expression in response to certain stimuli. Previously characterized as orphans, it was thought that they act independently of ligands to constitutively modulate gene expression [33, 81, 86-88], however this may no longer be the case since ligands such as UFAs have been discovered to bind the LBD of NR4A1 [75]. They can bind as monomers, homodimers, or heterodimers with each other or with RXR [81, 89-92]. The specific sequence they bind as monomers is called the NGFI-B response element (NBRE) and consists of 5'-A/TAAAGGTCA [93-95]. However as homodimers and NR4A heterodimers, they bind the Nur-responsive element (NurRE), which consists of a sequence motif found naturally occurring in the pro-opiomelanocortin (POMC) promoter [90]. Of the NR4A family, only NR4A1 and NR4A2 form heterodimers with RXR, which bind to the retinoic acid response element DR5 to mediate the function of RXR in retinoid signaling [91, 92].

The NR4A family is classified as immediate early response genes and can be induced by a wide variety of physiological signaling molecules to mediate their functions in proliferation, metabolism, inflammation, differentiation, apoptosis, survival, and DNA repair, among other functions [33, 96, 97]. They have an innate ability to respond quickly to these various stimuli, which include growth factors, stress, fatty acids, hormones, cytokines, neurotransmitters, prostaglandins, phorbol esters, calcium, and various apoptotic signals [33, 98-109]. In addition, they can also be induced by membrane depolarization, magnetic fields, and mechanical stress [33, 104, 110-112]. Importantly, the effects of the NR4A family are extremely context dependent and can vary depending on the specific tissue and cell type as well as experimental conditions. Moreover, their functions are heavily influenced by their expression levels, subcellular localization, posttranslational modifications, interactions with other transcription factors, and crosstalk with many signaling pathways [97].

Physiological roles

Main functions. The NR4A family is expressed in a variety of tissues, mainly in those that require higher levels of metabolism and energy. These include skeletal muscle, adipose, kidney, liver, T-cells, heart, and the brain [33, 92, 113-116]. The first major function identified for NR4A1 was its requirement for T-cell receptor (TCR)-mediated apoptosis in immature thymocytes and T-cell hybridomas [117, 118]. Both NR4A1 and NR4A3 are highly expressed during TCR-mediated cell death [117, 118], and inhibiting NR4A1 effectively blocks apoptosis in T-cell hybridomas. The lack of NR4A1 in

transgenic mice can also prevent the process of negative selection [119, 120]. In contrast, the presence of wild type Nr4a1 or Nr4a3 in mice enable cell death in thymocytes, resulting in reduced numbers of thymocytes and mature T cells [119-121]. A proposed mechanism by which NR4A1 mediates this process is via transcriptional activation of pro-apoptotic genes including Fas-ligand (FasL) [122]. In addition, both NR4A1 and NR4A3 may localize to the mitochondria and interact with Bcl-2 to induce apoptosis during the negative selection of T cells [97, 123]. Furthermore, calcium signaling was also found to be required for NR4A1-mediated apoptosis in T cells [124].

Compared to NR4A1, not as much is known about the physiological functions of NR4A2 and NR4A3. However, it is known that NR4A2 plays an important role in the synthesis of dopamine by mediating the transcriptional activation of tyrosine hydroxylase, an enzyme essential for dopamine synthesis [125]. Furthermore, NR4A2 is crucial to the normal development of the midbrain by ensuring the proper synthesis of dopaminergic neurons and facilitating neurotransmitter identity [126]. Interestingly, NR4A2 has been implicated in Parkinson's disease due to genetic mutations [127].

Neurological functions. In addition to the role of NR4A2 in dopamine synthesis, the NR4As appear to have neuroprotective functions as well as roles in learning as they are induced in the hippocampus during fear conditioning in mice [128]. In addition, NR4A1 was found to be required for object location while NR4A2 was important for recognition, long term memory, and object location [129]. NR4A1 has also been implicated in synaptic remodeling as well as a disease resulting from antipsychotic drugs [74, 130, 131]. Furthermore, exogenous NR4A1 expression can mediate the repair of damaged neurons after stroke wherein NR4A1 is normally decreased in neural cells lacking oxygen and glucose [132].

Inflammation and metabolism. The NR4A family has been found to play roles in steroidogenesis by regulating the expression of genes involved in the hypothalamic-pituitary-adrenal (HPA) axis, a complex network of glands important for many bodily functions including digestion, mood, and the immune system [133-135]. For example, corticotropin releasing hormone (CRH) and POMC expression are enhanced by NR4A1, resulting in activation of the HPA axis and downstream adrenal glucocorticoid synthesis [135]. The NR4As may also have roles in muscle, adipose, and macrophages as they are induced by various stimuli in these tissues. For example, all three NR4A receptors are rapidly induced by lipopolysaccharide (LPS) and more slowly induced by IFNγ in macrophages while only NR4A1 expression is enhanced by oxidized lipids and cytokines in these cells [136, 137]. Although NR4A1 is induced by inflammatory stimuli, it appears to play a protective role during inflammation as demonstrated by its involvement in T-cell development, Treg cell differentiation, and its anti-inflammatory effects in macrophages [138-140].

The NR4As are also induced in skeletal muscle during recovery from intense exercise [141]. NR4A1 is important for energy expenditure since knockdown of NR4A1 in C2C12 skeletal muscle cells resulted in decreased expression of genes involved in lipolysis and lipid homeostasis [142], suggesting a role for NR4A1 in treating obesity.

Similarly, the NR4As are induced during the differentiation of adipocytes, however their expression does not appear to be a requirement for differentiation to occur [96, 143]. These studies indicate that the NR4A family is important in regulating lipid and glucose metabolism, implicating them in the treatment of metabolism disorders.

Furthermore, the NR4A family was found to be upregulated in the livers of type I and type II diabetic mice [144, 145]. Knockout of NR4A1 in the liver of type II diabetic mice brought the elevated levels of glucose back to almost normal levels and also resulted in decreased expression of gluconeogenic genes [144, 145]. Consistent with this finding, overexpression of NR4A1 induced genes involved in gluconeogenesis and enhanced hepatic glucose production in mice [96]. NR4A1 overexpression can also increase LDL cholesterol and decrease HDL cholesterol while reducing triglyceride levels in the liver [146]. Other studies have shown that NR4A1-deficient mice develop insulin resistance in their skeletal muscle when fed a high-fat diet [147]. These mice also have decreased gene expression related to glucose utilization in skeletal muscle, whereas NR4A1 overexpression in C2C12 cells results in increased expression of genes involved in glucose and glycogen metabolism [147, 148]. This indicates a role for the NR4A family in the promotion of type II diabetes.

Cardiovascular system. In addition, the NR4As play roles in energy homeostasis and the vascular system. All three NR4As had increased expression in brown adipose tissue (BAT) during cold exposure, and NR4A1 is induced after β-adrenergic stimulation in brown adipocytes, although Nr4a1 deficient mice do not display abnormal nonshivering thermogenesis [149]. This lack of NR4A1 may be compensated by the potential redundancy of NR4A3 since NR4A3 is also induced in brown fat during cold exposure [149]. Interestingly, tissue-specific knockdown of *Nr4a3* in the third cerebral ventricle in mice resulted in decreased food consumption and body weight, suggesting a role for the NR4As in energy homeostasis by affecting food intake [96]. In terms of the vascular system, the NR4As are expressed in atherosclerotic lesions and have been shown to regulate genes involved in vascular remodeling processes such as proliferation and inflammation [96]. NR4A1 appears to play a protective role in cardiovascular disease since mice fed a high fat diet experienced decreases in atherosclerotic plaque formation, inflammation, and hepatic lipid deposition in response to NR4A1 overexpression [150-152]. In contrast, *Nr4a3* knockdown in mice decreases hypercholesterolemia-induced atherosclerosis, suggesting that NR4A3 may promote atherosclerosis [153].

NR4A knockout mice. Surprisingly, NR4A1-null mice do not display abnormal thymic and peripheral T cell death [133, 154]. Overall, they appear healthy with no noticeable phenotype or developmental problems [154]. The hypothalamic and pituitary systems also appear to function normally, as well as steroidogenesis [33, 133]. One explanation is the possible functional redundancy between the NR4A family wherein the other members may compensate for the lack of NR4A1. For example, it is known that NR4A3 is also capable of pro-apoptotic activity in thymocytes, and NR4A2 and NR4A3 also play roles in the HPA axis that could regulate HPA-related genes in place of NR4A1 [33, 123, 134, 135, 154, 155]. However, mice with both *Nr4a1* and *Nr4a3* knocked out quickly develop acute myeloid leukemia (AML) and die within 2 to 4 weeks postnatally

[156]. As a result of this double knockout, there was increased accumulation of hematopoietic stem cells. Expression of *NR4A1* and *NR4A3* was also found to be decreased in patients with AML [156].

Interestingly, mice that lack Nr4a2 develop to full-term but die at birth, seemingly due a defect in respiratory function. However, others report it is due to the lack of dopaminergic neurons in the midbrain with the observation of abnormal movements such as difficulty turning when the mice were placed on their backs as well as the inability to suckle [126, 157, 158]. Finally, *Nr4a3* knockout mice exhibit inner ear defects with impaired bi-directional circling behavior, which was associated with decreased endolymphatic fluid space in the ear canals [159, 160]. In addition, *Nr4a3*-null mice are more susceptible to limbic seizure activity due to excitotoxic glutamate receptor agonists [160]. In contrast, another study found that mice lacking Nr4a3 die around embryonic day 8.5 (E8.5) due to incomplete gastrulation [161].

Apoptotic function

Of interest is the translocation function of NR4A1 from the nucleus to the mitochondria to induce apoptosis. Although it appears the main function of NR4A1 is to exert pro-oncogenic and anti-apoptotic effects in cancer cell lines, there are several instances in which this is not the case. For example in lung and other cancer cell lines, addition of the retinoid CD437 (AHPN) induces apoptosis in a NR4A1-dependent manner (**Figure 1-2**) [162-170]. Many other stimuli have also been shown to induce nuclear export of NR4A1 to mediate apoptosis, including 5-fluorouracil, viruses, phorbol ester (TPA), butyrate, cadmium, and cytosporone B (Csn-B), among many others listed in **Table 1-2** [67]. These pro-apoptotic compounds may act by either inducing NR4A1 expression or by directly binding to NR4A1. For example, one study shows that the natural product Csn-B (derived from endophytic fungi) is able to directly interact with the LBD of NR4A1 to mediate NR4A1-dependent apoptosis through multiple mechanisms, including both transcription-dependent and -independent functions (**Figure 1-2**) [171, 172]. Csn-B treatment of gastric cancer cells resulted in enhanced NR4A1 expression, which was due in part to autoregulation in which NR4A1 was able to bind its own promoter to induce expression [171]. NR4A1 was also found to transcriptionally decrease the antiapoptotic protein BRE in response to Csn-B [172-174]. On the other hand, Csn-B induced translocation of NR4A1 to the mitochondria prior to activation of the apoptotic cascade [171]. This was the first evidence of a molecule that could act as a ligand to bind and activate NR4A1.

The mechanism by which NR4A1 mediates apoptosis involves directly binding to the anti-apoptotic protein Bcl-2 at the mitochondria and converting it to a pro-apoptotic protein. This conversion involves a conformational change of Bcl-2 that reveals its pro-apoptotic BH3 (Bcl-2 homology) domain [175]. Subsequently, cytochrome c is released and the intrinsic apoptotic cascade is evoked [168]. In addition to the mitochondria, NR4A1 may also localize to the endoplasmic reticulum (ER) to mediate apoptosis. Treatment of neuroblastoma cells with retinoid-related compound CD437 (AHPN)

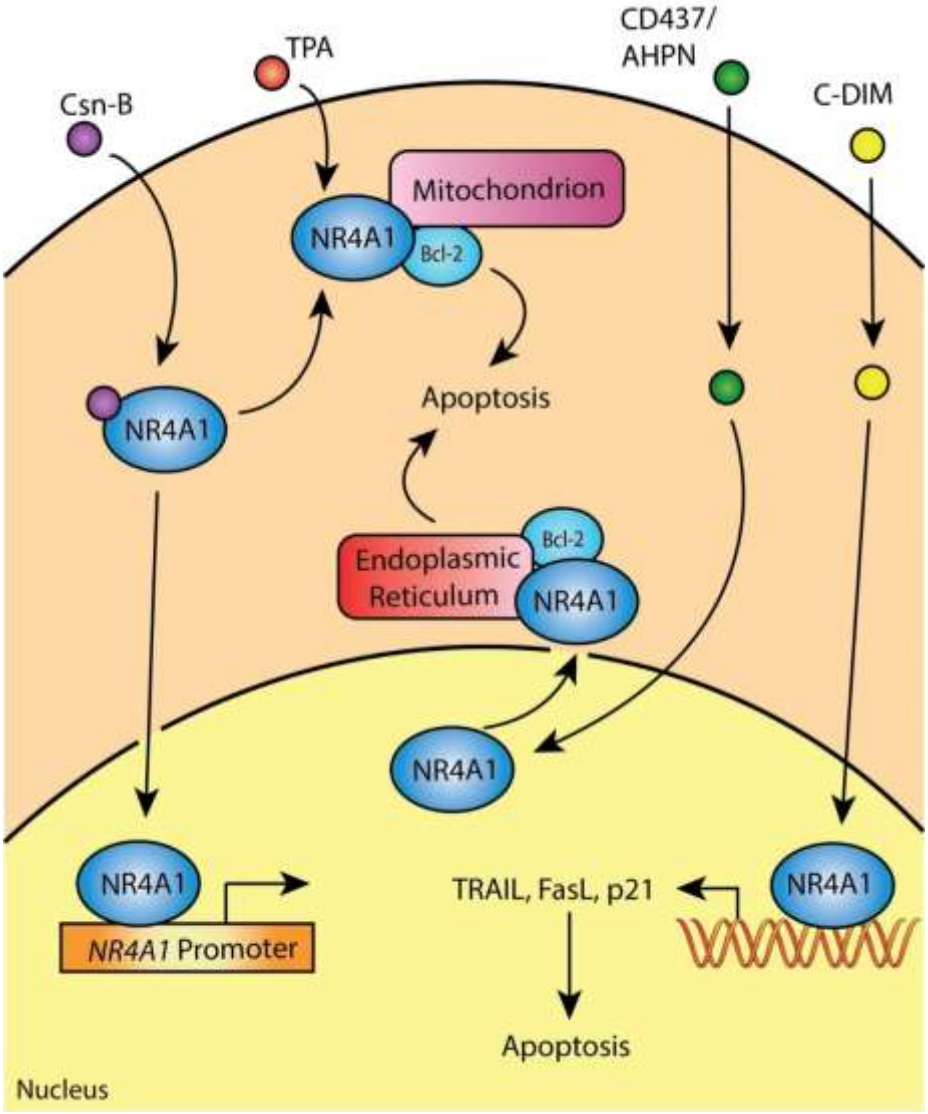

Figure 1-2. NR4A1 is induced by cytotoxic agents to mediate cell death.
NR4A1 can induce apoptosis in response to apoptotic compounds by either translocating to the mitochondria or to the endoplasmic reticulum and converts Bcl-2 to a proapoptotic protein. NR4A1 can also mediate apoptosis by transcriptionally activating genes involved in the cell death program.

Table 1-2. Compounds and apoptotic stimuli that induce Nur77 to mediate cell death, mainly through mitochondrial localization.

Inducer	Model system	References
N-butylidenephthalide (BP)	HCC	[176]
di-n-butyltin dichloride (DBTC) and tri-n-butyltin chloride (TBTC)	Rat thymocytes	[177]
Synthetic chenodeoxycholic acid derivatives	Gastric cancer	[178]
Cisplatin	Ovarian cancer	[179]
Butyrate	Colon cancer	[180]
Sulindac	Colon cancer	[180]
5-fluorouracil	Colon cancer	[180]
Phorbol ester 12- O-tetradecanoyl phorbol-13-acetate (TPA)	Prostate and gastric cancer	[168, 181]
Calcium ionophore A23187	Prostate cancer cells	[168]
Etoposide VP-16	Prostate and gastric cancer	[168, 181]
AHPN analog 6-[3-(1-adamantyl)-4- hydroxyphenyl]-3-chloro-2-naphthalenecarboxylic acid (MM11453)	Prostate cancer	[168]
Retinoid (Z)-4- [2-bromo-3-(5,6,7,8-tetrahydro-3,5,5,8,8- pentamethyl-2-naphthalenyl)propenoyl]benzoic acid (MM11384)	Prostate cancer	[168]
Sindbis virus	Primary B cells	[182]
Insulin-like growth factor-binding protein-3 (IGFBP-3)	Prostate cancer	[183]
Shikonin derivatives	Lung and cervical cancer	[184]
Cytosporone B (Csn-B) and related analogues	Gastric cancer	[171, 173]
9-cis-retinoic acid	Gastric cancer	[170]
Cadmium	Lung cancer	[185]
CD437 (AHPN)	Neuroblastoma and esophageal squamous carcinoma	[186]
C-DIMs	Pancreatic cancer	[187, 188]

induced NR4A1 nuclear export and interaction with Bcl-2 at the ER. This interaction led to Ca^{2+} release from the ER and subsequent ER stress, resulting in apoptosis via ER-mediated caspase-4 activation and ultimately activation of caspase-9 [186].

This apoptotic function of NR4A1 is currently being exploited for its therapeutic potential in cancer. One class of compounds called methylene-substituted diindolylmethanes (C-DIMs) looks especially promising as one group in particular has been studying the utilization of C-DIMs in treating cancer via NR4A1-mediated apoptosis [67]. C-DIMs are small lipophilic molecules synthetically derived from cruciferous vegetables and have been found to inhibit cell and tumor growth in many types of cancers both *in vitro* and *in vivo* [65, 170]. One C-DIM in particular, DIM-C-pPhOCH$_3$, induces apoptosis in several cancer types, and knockdown of NR4A1 inhibits this effect in pancreatic, colon, and bladder cancer cells [187, 188]. Several pro-apoptotic genes including TRAIL, FasL, and p21 were also induced, whereas NR4A1 knockdown abrogated this effect [67]. Interestingly, NR4A1 was not exported from the nucleus and did not translocate to the mitochondria during C-DIM-induced apoptosis, suggesting NR4A1 mediates apoptosis via transcriptional activation of these pro-apoptotic genes (**Figure 1-2**) [67].

On the other hand, the C-DIM analog DIM-C-pPhOH does not activate NR4A1. Instead, it induces apoptosis by preventing NR4A1 from transcriptionally activating anti-apoptotic genes such as survivin [67, 187]. Thus, C-DIMs exhibit anti-tumor properties by acting as both activators and deactivators of NR4A1 and take advantage of the differential activation of target genes dependent on the specific stimuli [67]. It is also possible to synthesize NR4A1 mimics. For example, one group has created a nanopeptide called NuBCP-9 (NR4A1-derived Bcl-2-converting peptide with 9 amino acids), which is capable of inducing apoptosis in cancer models *in vitro* and *in vivo* by replicating the action of NR4A1 and converting Bcl-2 to a pro-apoptotic molecule [189]. This NR4A1 mimic would be beneficial in the treatment of breast cancer since this cancer type has higher Bcl-2 expression [172]. Furthermore, the anticancer drug paclitaxel was found to mimic the effects of NR4A1 on Bcl-2 [190]. In all, many anti-tumorigenic compounds can induce NR4A1-mediated apoptosis through various mechanisms including the induction of NR4A1 expression and subsequent mitochondrial or ER targeting (CD437 and TPA), direct binding of compounds (Csn-B) with NR4A1 to activate apoptosis via genomic and non-genomic functions, and either activating or deactivating NR4A1 transcriptional activity (C-DIMs).

MicroRNAs

Discovery and nomenclature

Discovery. The first miRNA discovered was lin-4 in *Caenorhabditis elegans* (*C. elegans*) in 1993 [191]. Seven years passed before the second miRNA (let-7) was identified in 2000, also in *C. elegans* [192, 193]. Initially, it was thought that miRNAs

were specific to *C. elegans* until additional miRNAs were discovered in several other organisms, including humans [194, 195]. To catalog of all the miRNAs identified, a database called miRBase was created, with the most recent update released in 2014 (miRBase 21). This miRNA registry now contains 28,645 hairpin precursor miRNAs, which represents 35,828 mature miRNAs across 223 species (miRBase 21). Of these miRNAs, 1,881 precursors and 2,588 mature miRNAs belong to humans.

Nomenclature. For every miRNA identified, each is assigned a number corresponding to the order in which it was discovered. To specify the organism that the miRNA pertains to, three letters are added before the 'miR', such as hsa-miR-124 for *Homo sapiens* and mmu-miR-124 for *Mus musculus*. To denote the mature form of the miRNA, the 'r' is capitalized (miR-124), whereas a lower case 'r' (mir-124) indicates both the gene and precursor forms. When mature miRNAs have identical sequences but are processed from different precursor miRNAs, they are differentiated by adding a number to the end of their name, such as miR-124-1 and miR-124-2. Conversely, mature miRNA sequences that differ by one or two nucleotides are denoted by a letter, such as miR-34a and miR-34b. In addition, miRNAs are processed from precursor miRNAs into two complementary mature miRNAs where one is on the 3′ strand and the other resides on the 5′ strand. These can be denoted by adding '3p' or '5p', such as miR-124-3p and miR-124-5p. One version may be predominantly expressed over the other, and when the dominant version is known, it keeps the common name (miR-124), whereas the less dominant one is indicated with an asterisk (miR-124*) [196-199].

Biogenesis and function

miRNAs are non-coding RNAs consisting of 18 to 23 nucleotides that regulate gene expression by binding to the 3′ untranslated region (3′UTR) of their target gene and degrading the mRNA, thereby preventing its translation into protein [200, 201]. About half of all miRNAs reside in non-protein coding genes, while the other half are usually within the introns of protein coding transcripts [202]. RNA polymerase II transcribes them into long primary miRNAs (pri-miRNA) that resemble a stem-loop structure, which are then cleaved by the enzyme Drosha before becoming precursor miRNA (pre-miRNA) [203]. These pre-miRNAs are then exported into the cytoplasm via exportin-5 in a RanGTP-dependent fashion where they are further cleaved by Dicer into mature miRNAs, as shown in **Figure 1-3** [204-206]. During the final stage of biogenesis, the mature miRNAs consist of two complementary strands that are separated with the more stable passenger strand being degraded and the unstable guide strand being incorporated into the RNAi induced silencing complex (RISC) [195, 207, 208]. This complex is then able to bind the 3′UTR of its target mRNA in the seed region, which consists of 6 to 8 complementary nucleotides. Although the 3′UTR is the main target site, miRNAs may also bind the 5′UTR as well as the coding sequence [209, 210].

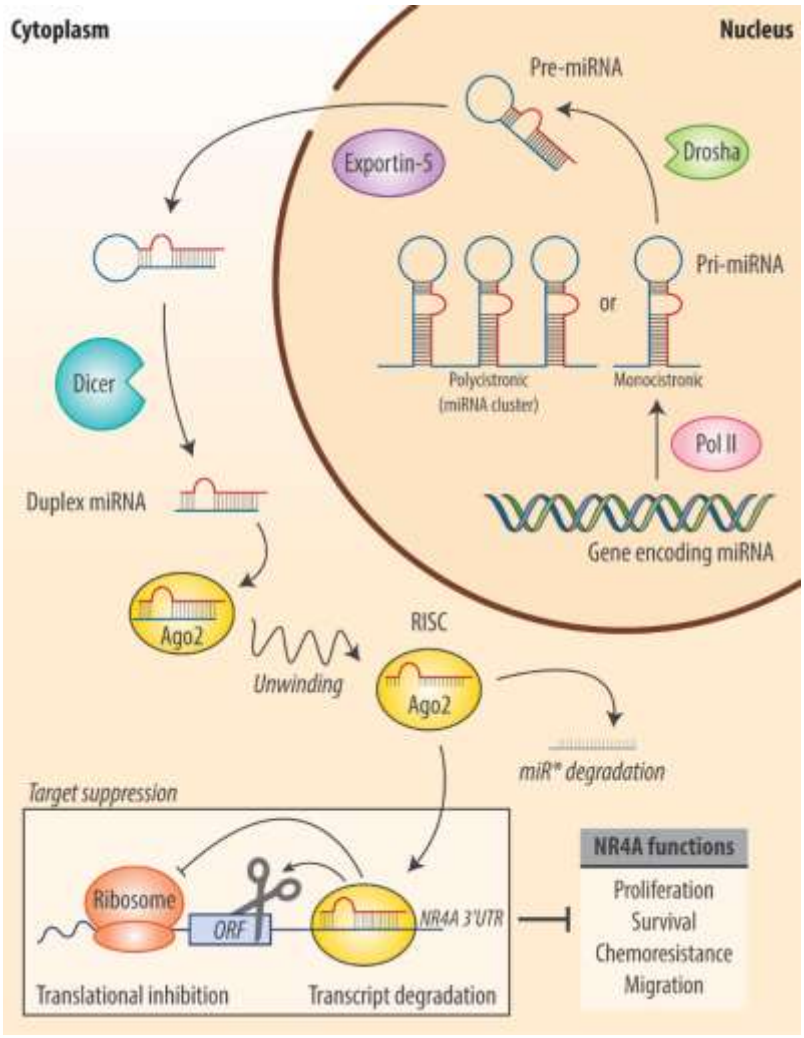

Figure 1-3. miRNA biogenesis and function.
miRNAs are transcribed by RNA polymerase II (Pol II) into either polycistronic or monocistronic pri-miRNA, which are cleaved by Drosha into pre-miRNA. Exportin-5 mediates the translocation of pre-miRNA into the cytoplasm where it is cleaved by Dicer into mature miRNA. A single strand of the mature miRNA is incorporated into the RISC complex, which can then bind to the 3'UTR of target genes to mediate gene suppression. This can lead to inhibition of NR4A oncogenic functions such as proliferation, survival, chemoresistance, and migration.

Therapeutic potential

miRNAs are important regulators of almost every cellular process and are estimated to regulate 60% of the human genome [211]. They are able to form intricate networks wherein a single miRNA can target hundreds of genes, while a single gene may be targeted by numerous miRNAs. This complex regulation has the potential to have profound effects on biological processes. For example, miRNAs have been shown to play important roles in cancer where they can act as tumor suppressors by targeting oncogenes or as oncomiRs by targeting tumor suppressors [212, 213]. Furthermore, many cancers exhibit aberrant miRNA expression where the tumor suppressor-like miRNAs are downregulated, most likely due to hypermethylation of the promoter [214].

In addition to miRNAs acting as biomarkers, they have also seen increased potential for use as therapeutic agents such as miRNA replacement therapy using miRNA mimics [215]. For instance, the miR-34 family harbors strong inhibitory effects on tumor growth in a variety of cancers. It exerts these effects through a number of mechanisms including the direct suppression of mitogenic genes (cyclin E2, E2F3, and CDK4) as well as the anti-apoptotic gene SIRT1, allowing for increased expression of the apoptotic genes p53, p21, and PUMA [216]. In addition, p53 induces miR-34 expression, resulting in a positive feedback loop [217, 218]. A cancer therapy utilizing the power of miR-34 is currently being tested where a miR-34 mimic is encapsulated in a liposome to allow for more targeted delivery in a range of cancers and is currently being tested in phase I clinical trials. This is the first miRNA to enter clinical trials, and the results thus far appear promising [219]. Many other tumor suppressor-like miRNAs exist with similar effects to miR-34, suggesting the potential for further exploration of miRNA replacement therapy if the miR-34 mimic proves successful.

On the other hand, miRNAs may play an oncogenic role such as the miR-17-92 cluster promoting tumorigenesis in a wide range of cancers [220]. In addition to cancer, miRNAs can have important functions in metabolic disorders, autoimmune diseases, genetic diseases, and infectious diseases [195]. Of importance, the viral replication of hepatitis C virus (HCV) was found to be dependent on miR-122 [221]. In order to block miR-122 function, a drug was developed (SPC3649) to effectively bind the miRNA in a locked nucleic acid fashion. After successful experimentation in chimpanzees, the drug is currently being tested in phase II clinical trials in humans [222]. If successful, this will be the first miRNA-based therapy for HCV [195].

Hypothesis and Specific Aims

Studies have shown that NR4A1 is upregulated in many solid tumors, and this overexpression can lead to increased cell proliferation in cancer cell lines, indicating an oncogenic role for this nuclear receptor. Our overall hypothesis is that miRNAs can regulate the expression and downstream function of NR4A1, as depicted in **Figure 1-3**. We theorize that one of the causes for upregulation of NR4A1 in cancer is due to downregulation of tumor suppressor-like miRNA regulators of NR4A1.

In addition to this first project, we decided to explore the potential function of NR4A1 during the differentiation of skeletal muscle. Previous studies found that NR4A1 expression increases during skeletal muscle differentiation. Thus, we hypothesize that NR4A1 is playing a pro-myogenic role during this process. To investigate these hypotheses, we proposed the following aims:

1. Elucidate the miRNAs that are directly regulating NR4A1 and their effects on the proliferative function of NR4A1 in pediatric cancer cell lines.

2. Determine the importance of NR4A1 in skeletal muscle differentiation.

Significance of Study

NR4A1 has been mainly studied in adult cancers, with little research on its role in pediatric cancers. Although we propose to focus on pediatric cancer types, this novel study of the interaction between miRNAs and NR4A1 could have therapeutic potential in both adult and pediatric cancers. NR4A1 is an ideal candidate for a therapeutic target and a potential biomarker in cancer. On the other hand, miRNAs can act as tumor suppressors and are useful as prognostic markers as well as miRNA mimic therapy to treat a variety of cancers.

The discovery of miRNAs that target NR4A1 will add a new aspect to NR4A1 regulation. This study could demonstrate clinical translation through the use of *in vivo* models in which miRNAs are used as therapy for the pediatric tumors in which NR4A1 is aberrantly amplified. The majority of published research on NR4A1 as a therapeutic target involves utilizing agonists to induce NR4A1-mediated apoptosis, but none have delved into using miRNAs to influence NR4A1 expression and genomic actions.

In addition, our initial studies show that differentiation of skeletal muscle cells is severely delayed upon knockdown of NR4A1. This data supports previous studies showing the importance of NR4A1 in the muscle mass of mice. This information could be useful in terms of cancer therapy since malignancies such as rhabdomyosarcoma (RMS) form as a result of cells failing to differentiate. Furthermore, muscle-related diseases can arise such as muscular dystrophy, a disease that is prevalent in younger populations and is characterized by loss of muscle mass and progressive weakness. Determining the role of NR4A1 during skeletal muscle differentiation may help understand the formation of RMS as well as diseases related to muscle.

CHAPTER 2. THE REGULATORY EFFECTS OF MICRORNAS ON NR4A1 IN CANCER

Introduction

NR4A1, a member of the NR4A family of nuclear receptors, is known for its oncogenic effects in cancer cells. These effects are heavily dependent on its role as a transcription factor to promote expression of genes leading to cell proliferation and survival. The expression and function of NR4A1 are regulated by post-translational modifications and protein-protein interactions, as well as transcriptional regulation by other transcription factors. NR4A1 is aberrantly expressed in many cancers, and one way to therapeutically modulate its expression is through microRNA (miRNA) replacement therapy. This chapter will explore the ways in which miRNAs may be affecting NR4A1 expression and function in the context of cancer. The role of NR4A1 in cancer will first be discussed, followed by the many different ways it may be regulated by miRNAs. Both direct and indirect effects will be detailed since there are not many miRNAs shown to target NR4A1 directly. miRNAs may be targeting NR4A1 indirectly by preventing the expression of genes that would normally interact and regulate NR4A1 via transcriptional regulation, post-translational modifications, and protein-protein interactions. These regulatory networks will be related back to cancer and hypothetical situations will be discussed on how they may affect tumor formation and progression. It is important to understand these networks in order to determine the best possible way for treating cancers in which NR4A1 is aberrantly expressed and plays oncogenic roles.

NR4A1 in Cancer

In many adult cancers, NR4A1 is overexpressed and appears to play a proliferative role. Bladder [223, 224], breast [187, 225-228], colon [180, 188, 228-231], liver [176, 231, 232], pancreatic [233, 234], prostate [235], ovarian [228, 236], and lung [167, 228, 237, 238] cancer all display increased NR4A1 compared to their normal counterparts, with melanoma [228] expressing the highest levels of NR4A1. The transactivation and DNA-binding domains of NR4A1 appear to be the culprit for its proliferative function in these cancers. Through transcriptional upregulation of genes involved in cell cycle progression and inhibition of apoptosis, NR4A1 can thereby exert its mitogenic effects. For example in melanoma and lung cancer, NR4A1 mediates cell cycle progression and proliferation as well as angiogenesis, which was dependent on its transactivation and DNA binding ability [167, 239]. Furthermore, knockdown studies of NR4A1 in many cancer cell lines resulted in decreased cell growth and angiogenesis and increased apoptosis, providing further evidence for the role of NR4A1 as an oncogenic factor [67]. On the other hand, NR4A1 is downregulated in acute myeloid leukemia (AML), and *Nr4a1*/*Nr4a3* knockout mice quickly develop AML, leading to the suspicion that NR4A1 is playing a tumor suppressive role in this blood cancer [156]. This seems to contradict its role in solid tumors, but it is more understandable after realizing that NR4A1 possesses the ability to exert opposing roles in different tissues and cell types.

NR4A1 can also wield its effects by other means, such as interacting with proteins from a multitude of signaling pathways, as well as phosphorylation by kinases to either promote or inhibit its translocation to the cytoplasm. As discussed below, miRNAs also factor into this regulation of NR4A1 activity by both direct and indirect effects, thereby affecting cancer progression in a myriad of ways.

miRNAs That Directly Target *NR4A1*

Currently there is a shortage of miRNAs that have been proven to target *NR4A1*. According to miRTarBase there are no miRNAs that have been validated to target *NR4A1* using strong evidence consisting of reporter assays, western blots, and qPCR. There are a total of 6 miRNAs shown to target *NR4A1* using less strong evidence such as microarrays and next-generation sequencing (NGS). For example, one study used a microarray and found that HeLa cells transfected with miR-124 had decreased *NR4A1* mRNA [240]. Another study also used a microarray and showed that rno-miR-290 targets *Nr4a1* in the rat brain [241]. However, there is one recent study that does validate the targeting of *NR4A1* by miR-124, miR-15a, and miR-224 through the use of reporter assays, western blot, and qPCR. This study also found that miR-124 can suppress the transcriptional activity of NR4A1 in Daoy medulloblastoma cells. When miR-124 was exogenously expressed in these cells, proliferation and viability were significantly decreased [242]. Although miR-124 is predicted to target many other genes, it is possible that it is causing these anti-tumor effects through the suppression of *NR4A1*. miR-124 is an interesting miRNA because it is the most abundant miRNA in the brain with a role in neuronal differentiation. It is a tumor suppressor in many cancer types, several of which express aberrantly low levels of this miRNA. Since NR4A1 is commonly upregulated in cancer, it is easy to see how this aberrant upregulation could be due to the downregulation of miRNAs that target it such as miR-124.

miRNAs That Indirectly Affect NR4A1 Expression and Function

Proteins that modulate *NR4A1* mRNA expression

There are several ways in which miRNAs may indirectly affect NR4A1 expression and function. One way is by targeting proteins that modulate *NR4A1* mRNA expression. For example, the histone acetyltransferase p300 can acetylate *NR4A1*, thereby enhancing its expression in HepG2 and HeLa cells [243]. In addition, the transcription factor AP-1 was shown to bind the promoter of *NR4A1* and induce its expression in colon cancer cells after exogenous β-catenin expression [230]. β-catenin was also found to increase *NR4A1* transcription through hypoxia-inducible factor 1-alpha (HIF-1α) in colon cancer cells in response to hypoxic conditions [244]. β-catenin is aberrantly activated in 90% of colon tumors and acts as an oncogene in this cancer [230], therefore it would appear that β-catenin is exerting its proliferative effects partly through its induction of *NR4A1*. HIF-1α was also found to bind to the promoter of *NR4A1* and promote its

expression in several cancer cell types including renal cell carcinoma, neuroblastoma [245], hepatocellular carcinoma (HCC), cervical cancer, and breast cancer [246]. *EP300* (encodes p300), *FOS* and *JUN* (comprises AP-1), *CTNNB1* (encodes β-catenin), and *HIF1A* are targeted and repressed by many miRNAs as listed in **Table 2-1**. Upon repression of these genes by miRNAs, *NR4A1* expression would also theoretically decrease. On the other hand, HDAC1 is able to repress *NR4A1* in HepG2 and HeLa cells [243]. Therefore any miRNAs that target and suppress *HDAC1* would result in enhanced *NR4A1* expression.

Proteins that directly bind NR4A1

RXR. Another way miRNAs may indirectly affect NR4A1 is by targeting binding partners of NR4A1 that in turn affect its localization and function, as depicted in **Figure 2-1**. One of the most complex interactions may be with the retinoid X receptor (RXR). Depending on experimental conditions and external stimuli, RXR may heterodimerize with NR4A1 and either bind to DNA to modulate gene expression or localize to the mitochondria to induce apoptosis. When prostate and lung cancer cells were treated with apoptosis-inducing agents 3-Cl-AHPC and TPA (12-O-tetradecanoyl-13-phorbol acetate), RXRα formed a heterodimer with NR4A1 and translocated to the mitochondria to induce apoptosis [162]. However, translocation of this heterodimer and subsequent apoptosis were inhibited by the RXR ligand 9-cis-retinoic acid (9-cis-RA) [162]. This RXR ligand along with RXR-selective retinoids, SR11246 and SR11345, strongly promote RXR/NR4A1 heterodimer binding to the retinoic acid response element βRARE [91, 162, 247, 248], although NR4A1 can also increase RA response element transcription independent of retinoic acid [247]. Contrary to this finding, one group found that 9-cis-RA actually enhanced RXR/NR4A1 dimerization and translocation to the mitochondria, along with subsequent apoptosis [249]. This group also found that NR4A1 binding to RXR could suppress p300-mediated RXR acetylation, thereby decreasing the transcriptional and mitogenic activity of RXR [249]. It appears that the interactions and effects of NR4A1/RXR heterodimerization are dependent on many factors, and that seemingly contradictory findings may be due to differences in cell types among other factors. There are only a few miRNAs that directly target *RXRA*, including miR-128 and miR-574 [250, 251]. Therefore it is possible that expression of these miRNAs result in the opposite effects described here.

COUP-TF. In addition to RXR, the COUP-TF (chicken ovalbumin upstream promoter-transcription factor) orphan receptors can also bind with NR4A1 and have varying effects. In the absence of ligand, COUP-TFs bind RAREs and inhibit their transcription, leading to decreased RARB and increased cell growth, at least in breast and lung cancer. However in the presence of RA, COUP-TF has no effect on βRARE transcription and therefore enhances the anti-tumor effects of RA, leading to the conclusion that COUP-TF sensitizes cancer cells to RA. In lung cancer cells, NR4A1 heterodimerizes with COUP-TF and prevents binding to the βRARE, thereby desensitizing the cells to RA. On the other hand, COUP-TF can prevent NR4A1/RXR heterodimer binding to the βRARE via protein-protein interaction. Furthermore, COUP-

Table 2-1. Effects of Nur77 regulatory networks on cancer and potential implications by miRNAs.

Functional relationship to NR4A	Gene (common name)	Effect on NR4A/functional consequence	Oncogenic effect?	Cancer type/disease	miRNAs	miRNA references
Modulates Nur77 mRNA expression	*EP300* (p300)	Promotes expression via acetylation	Yes	Liver, cervical	20a, 26a, 106b~25, 132, 150, 574	[252-257]
	Fos/Jun (AP-1)	Promotes expression by directly binding to promoter	Yes	Colon	101, 181b, 155	[258-263]
	HIF1A (HIF-1α)	Promotes expression by directly binding to promoter	Yes	Neuroblastoma, cervical, breast, liver	17~92, 18a, 20b, 31, 93, 138, 155, 199ab, 206, 210, 335, 338, 519c	[264-279]
	CTNNB1 (β-catenin)	Promotes expression via activation of AP-1	Yes	Colon	34, 185, 200abc, 203, 214, 434, 680, 690	[280-286]
	HDAC1	Suppresses expression	No	Liver, cervical	34a, 449ab, 874	[287-294]
Binds Nur77 protein	*RXRA* (RXRα)	RXR/Nur77 heterodimer either activates transcription or translocates to mitochondria to induce apoptosis	Yes and No	Lung, pancreatic	128, 574	[250, 251]
	NR2F2 (COUP-TF)	Binds and prevents Nur77 from binding to RXR, thereby resensitizing cells to RA	Yes and No	Lung	194, 302a	[295, 296]
	CHD1L	Inhibits mitochondrial targeting	Yes	Liver	None	
	XRCC5 (Ku80)	Nur77 prevents Ku80-mediated DNA repair	No	Liver	31, 526b	[297, 298]
	TP53 (p53)	Nur77 blocks p53 transcriptional activity/promotes p53-mediated apoptosis	Yes and No	Lung, liver, bone	25, 30d, 125, 150, 375, 504, 1285	[299-305]
	STK11 (LKB1)	Nur77 prevents LKB1 from suppressing mTOR	Yes	Cervical	155	[306]
	PIN1 (Pin1)	Pin1 promotes Nur77 transcriptional activity	Yes	Cervical	140, 200bc, 296	[307-309]

Table 2-1. Continued.

Functional relationship to NR4A	Gene (common name)	Effect on NR4A/functional consequence	Oncogenic effect?	Cancer type/disease	miRNAs	miRNA references
	EP300 (p300)	Nur77 either inhibits p300 or forms complex to promote transcription	Yes and No	Cervical, breast, lung	20a, 26a, 106b~25, 132, 150, 574	[252-257]
	CTNNB1 (β-catenin)	Nur77 inhibits β-catenin function	No	Colon	34, 185, 200abc, 203, 214, 434, 680, 690	[280-286]
	NDRG1	Competitively binds Nur77 and prevents it from inducing β-catenin degradation	Yes	Liver	182, 769	[310, 311]
	VHL (pVHL)	Nur77 inhibits the pVHL-mediated ubiquitination of HIF-1α	Yes	Neuroblastoma, renal cell carcinoma	21	[312]
	NR0B2 (SHP)	SHP blocks Nur77 from transcriptionally mediating cell death upon treatment with an apoptosis-inducer	Yes	Liver	141, 378g, 4649	[313-315]
	PRKCA (PKC)	Nur77 prevents PKC from activating AP-1 and NF-kB	No	Acute T cell leukemia	24-2	[316]
	PML	Inhibits Nur77 transcriptional activity	No	osteosarcoma	None	
Phosphorylates Nur77	*AKT1* (Akt)	Nur77 mitochondrial targeting is blocked by Akt	Yes	Gastric	100, 105, 133b, 143, 149, 342	[317-322]
	JNK	JNK induces Nur77 mitochondrial targeting and inhibits its transcriptional activity	No	Lung, prostate, breast, glioma, ovarian, oral squamous cell carcinoma	92a	[323]
	PRKDC (DNA-PKcs)	Nur77 induces apoptosis upon ionizing radiation treatment, which is dependent on its phosphorylation by DNA-PKcs	No	Liver	101	[324]

Table 2-1. Continued.

Functional relationship to NR4A	Gene (common name)	Effect on NR4A/functional consequence	Oncogenic effect?	Cancer type/disease	miRNAs	miRNA references
	GSK3B (GSK-3β)	Nur77 is blocked from inhibiting B-catenin transcriptional activity upon phosphorylation by GSK-3B	Yes	Colon	26a, 346	[325, 326]
Transcriptional target of Nur77	*CCND2* (cyclin D2)	Promotes proliferation via cyclin D2	Yes	Cervical	1, let-7a, 15b, 16, 26a, 29abc, 30c, 98, 124a, 145, 182, 195, 198, 204, 206, 302b, 340, 375, 497, 610	[327-350]
	TXNDC5	Promotes lower stress levels and therefore increased survival	Yes	Pancreatic	200b	[351]
	BIRC5 (survivin)	Promotes survival	Yes	Pancreatic	16, 195~497, 203, 218, 542, 708	[352-361]
	E2F1	TPA induces Nur77, resulting in increased E2F1 and apoptosis; Pin1 promotes Nur77 to transcriptionally activate E2F1, resulting in increased proliferation	Yes and No	Prostate, cervical	106a, 136, 149, 205, 223, 320, 326, 329, 330, 331, 342, 362, 493, 603	[321, 362-374]
	CDK4	Nur77 activates CDK4 following induction by bile acids	Yes	Liver, colon	1, 34a, 124, 188, 195, 206, 486, 506, 613	[341, 375-387]
	BRE	Nur77 activates BRE following induction by bile acids	Yes	Liver, colon	None	
	RBBP8	Nur77 activates RBBP8 following induction by bile acids	Yes	Liver, colon	19ab, 335	[388, 389]
	MAP4K5	Nur77 activates MAP4K5 following induction by bile acids	Yes	Liver, colon	None	
	STAT5A	Nur77 activates STAT5A following induction by bile acids	Yes	Liver, colon	141, 222, 223, 1469	[390-393]

Table 2-1. Continued.

Functional relationship to NR4A	Gene (common name)	Effect on NR4A/functional consequence	Oncogenic effect?	Cancer type/disease	miRNAs	miRNA references
	BID	Nur77 activates BID following induction by bile acids	No	Liver, colon	None	
	POMC	Nur77 promotes POMC expression	Yes	Cushing's disease	None	
	CITED1	Nur77 represses CITED1, allowing Wnt activation	Yes	Melanoma	None	
	DACT1	Nur77 represses DACT1, allowing Wnt activation	Yes	Melanoma	None	
	MYC	Nur77 represses MYC	No	Acute myeloid leukemia	let-7g, 24, 34abc, 126, 135b, 145, 185, 320b, 487b, 494, 744	[394-408]

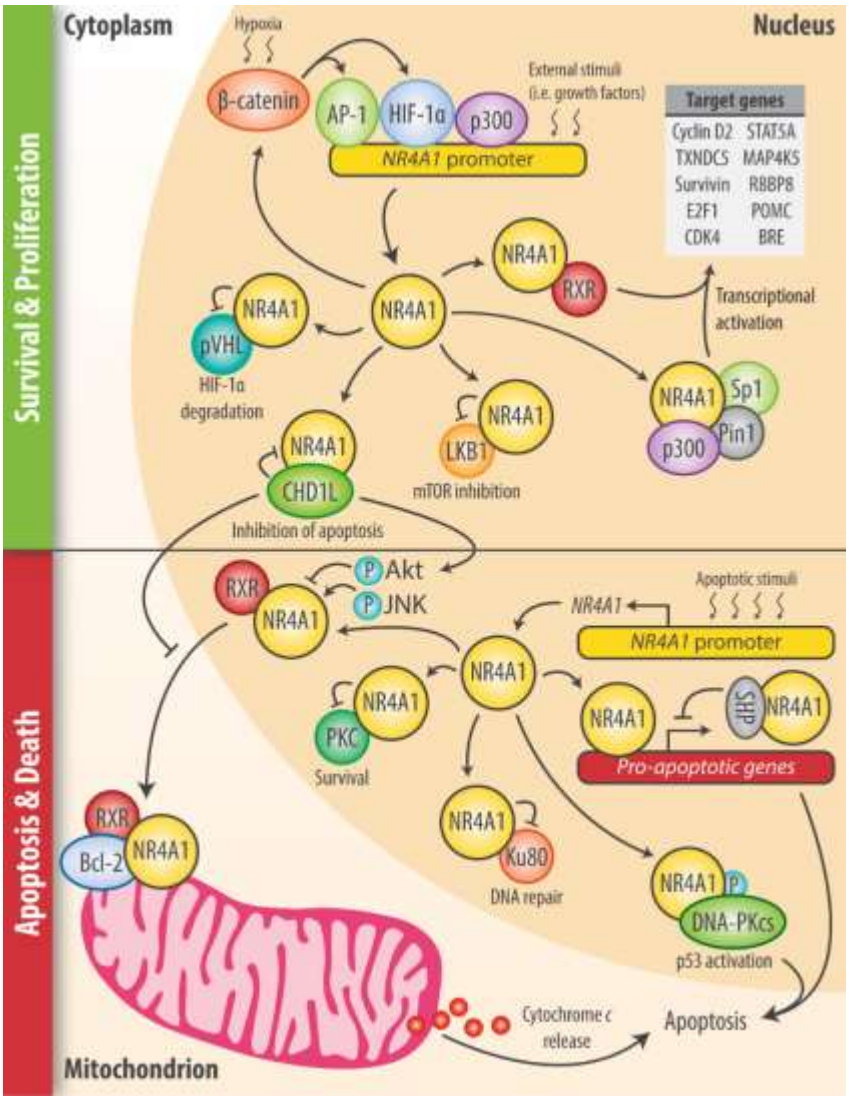

Figure 2-1. NR4A1 mediates cell proliferation and survival in addition to cell death.

The top panel depicts genes that transcriptionally activate *NR4A1*, leading to NR4A1 interactions that ultimately promote cell proliferation and survival. In contrast, the bottom panel details the actions of NR4A1 in response to apoptotic stimuli, resulting in translocation of NR4A1 to the mitochondria and induction of apoptosis.

TF expression was positively correlated with RA sensitivity, while NR4A1 expression was associated with retinoid resistance in lung cancer cells. This suggests NR4A1 is responsible for RA resistance, leading to enhanced cell proliferation [247]. miRNAs capable of directly binding and suppressing *NR2F2* (encodes COUP-TF) include miR-194 and miR-302a. COUP-TF suppresses osteoblastic differentiation, and overexpression of miR-194 and miR-302a was found to rescue this effect [295, 296]. This leads us to speculate that expression of these miRNAs would result in the desensitization of cancer cells to RA treatment mediated by NR4A1.

CHD1L. The next gene that physically interacts with NR4A1 is CHD1L. This protein acts as an oncogene in hepatocellular carcinoma and can bind with NR4A1 to prevent its translocation to the mitochondria and subsequent apoptosis upon treatment with staurosporine [409]. Unfortunately, there are no miRNAs that have been validated to target CHD1L. However, CHD1L interacts with Ku70 and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) to enhance chromatin remodeling and DNA repair [410, 411], although its elevated levels in cancer surprisingly lead to increased DNA damage. The DNA-PK complex is essential for mediating double-strand break repair and comprises DNA-PKcs and Ku70/Ku80. Since NR4A1 is known to interact with Ku80 to suppress DNA repair [232] and has also been shown to bind CHD1L, it is possible that NR4A1 inhibits DNA repair via protein-protein interactions with Ku80 and/or CHD1L. There are presently two miRNAs shown to target *XRCC5* (encodes Ku80), including miR-31 and miR-526b [297, 298]. Therefore expression of these miRNAs may lead to increased DNA damage, which would most likely lead to cell death.

p53. One of the more significant binding partners of NR4A1 is the tumor suppressor p53, and similar to some of its previously discussed binding partners, this interaction can lead to contradictory outcomes. NR4A1 can play an oncogenic role by binding to p53 and preventing p300 acetylation of p53, resulting in suppressed p53 transcriptional activity [412]. In addition, NR4A1 can circuitously promote oncogenic mTOR signaling by inhibiting p53-mediated transcription of sestrin-2, an activator of AMPK, which is an inhibitor of mTOR [238, 413]. However in the presence of DNA-PKcs, NR4A1 is phosphorylated by DNA-PKcs and becomes an activator of p53 transcriptional activity via phosphorylation of p53 by DNA-PKcs. In addition to increasing p53 transactivation, NR4A1 heterodimerization with p53 can enhance p53-mediated apoptosis as well as p53 protein stability by blocking ubiquitination and subsequent degradation of p53 by MDM2 [412]. As listed in **Table 2-1**, many miRNAs have been found to target *TP53*. Since p53 is a tumor suppressor, this would imply that any miRNA that targets it is likely an oncomiR.

LKB1. Another way in which NR4A1 promotes mTOR signaling is by inhibiting LKB1. NR4A1 binds LKB1 and blocks it from activating AMPK, thereby preventing AMPK from inhibiting mTOR. This leads to enhanced mTOR signaling and therefore increased proliferation, survival, and angiogenesis [414]. Interestingly, miR-155 was found to promote proliferation of cervical cancer cells by targeting *STK11* (encodes LKB1), therefore acting as an oncomiR [306].

Pin1 and p300. Additional proteins that interact with NR4A1 include p300 and Pin1. NR4A1 isomerization and increased protein stability is mediated by Pin1 and enhances recruitment of p300, thereby increasing the transcriptional activity of NR4A1 and its downstream proliferative effects [415]. NR4A1 can also form a complex with p300 and Sp1 to promote expression of proliferative and prosurvival genes such as survivin in lung cancer cells [238]. On the other hand, NR4A1 can directly interact with p300 and block its ability to acetylate transcription factors, thereby repressing their transcriptional activity and resulting in decreased proliferation in breast cancer cells [416]. Five miRNAs target *EP300*, including miR-20a [252], miR-132 [256], miR-150 [254], and miR-574 [253], as well as the miR-106b~25 cluster [255]. In addition, miR-200b [307], miR-200c [308], and miR-296 [309] were found to target *PIN1*. All of these miRNAs, with the exception of miR-20a and the miR-106b~25 cluster, play mostly tumor suppressive roles. Therefore it would make sense that expression of these miRNAs would have a negative impact on NR4A1 function via targeting of *EP300* and *PIN1*.

β-catenin. The proto-oncogene β-catenin is often mutated in cancer and interacts with transcription factors to promote expression of mitogenic genes. NR4A1 acts as a negative regulator of β-catenin function either by inducing its degradation in the cytoplasm [417] or by blocking its interaction with the transcription factor TCF4 and promoting recruitment of corepressors, thereby suppressing its transcriptional activity in colon cancer [418]. In contrast, NR4A1 was found to activate and stabilize β-catenin protein and prevent its degradation under hypoxic conditions via NR4A1-mediated activation of the Akt pathway. As mentioned, β-catenin can promote NR4A1 expression, thereby forming a positive feedback loop that promotes the proliferation of colon cancer cells [244]. *CTNNB1* (encodes β-catenin) is targeted by miR-34 [280, 419], miR-200a [282-284], and miR-214 [285, 286], all of which act as tumor suppressors in several cancer types. Five other miRNAs have also been shown to directly target *CTNNB1* [281], and expression of these miRNAs may either relieve NR4A1 of its inhibitory effect on β-catenin, or suppress the NR4A1-mediated activation of β-catenin induced by hypoxia.

NDRG1. N-myc downstream regulated gene 1 (NDRG1) acts as an oncogene in hepatocellular carcinoma by promoting β-catenin accumulation. It does so by competitively binding with glycogen synthase kinase-3β (GSK-3β) and NR4A1 individually, thus preventing β-catenin degradation by GSK-3β and NR4A1. This inhibitory mechanism by NDRG1 results in upregulation of downstream oncogenic genes [420]. miR-182 acts as an oncomiR in prostate cancer [310] while miR-769 [311] plays a tumor suppressive role in breast cancer, both of which mediate their effects by directly targeting *NDRG1*. However in HCC, expression of these miRNAs would presumably result in increased β-catenin degradation via NR4A1 and GSK-3β, thus leading to anti-proliferative effects.

SHP. Small heterodimer partner (SHP) commonly inhibits nuclear receptor function via physical interaction and was found to bind and suppress NR4A1 transcriptional activity. When HCC cells were treated with an inducer of apoptosis, SHP prevented NR4A1 from transcriptionally mediating cell death [421]. There are three miRNAs that target *NR0B2* (encodes SHP), including miR-141 [313], miR-378g [314],

and miR-4649 [315], expression of which would theoretically lead to enhanced apoptosis mediated by NR4A1.

PKC. As mentioned briefly, NR4A1 appears to be a tumor suppressor in leukemia. One way NR4A1 may mediate this effect is by interacting with and inhibiting protein kinase C (PKC). In Jurkat leukemic T cells, the LBD of NR4A1 binds PKC and prevents it from activating AP-1 and NF-kB [422]. The activation of NF-kB by PKC can lead to induction of anti-apoptotic genes, and since NR4A1 is known to induce apoptosis in T cells, its inhibition on PKC may be necessary in order for apoptosis to occur. miR-24-2 is the only miRNA that has been shown to directly target *PRKCA* (PKC) and was found to decrease cell survival in breast cancer cells via PKC inhibition [316].

PML. Another way NR4A1 is involved in leukemia is through its interaction with the promyelocytic leukemia gene, PML [423]. Patients with acute promyelocytic leukemia commonly have a chromosomal translocation that results in fusion genes encoding PML-RARα and RARα-PML [424]. PML is well known for its ability to inhibit cell proliferation and to act as a tumor suppressor *in vivo* [425-429]. PML was also found to directly interact with NR4A1 in its DBD domain and prevent its transcriptional activity in osteosarcoma cells [423]. Thus far there have not been any miRNAs found to target *PML*, but hypothetically any miRNA that suppresses PML would result in increased transcriptional activity by NR4A1.

VHL. Lastly, the tumor suppressor von Hippel-Lindau (pVHL) is an E3 ubiquitin ligase that forms a multimeric complex to degrade proteins and has been implicated in several diseases as a result of mutations in pVHL. One of its target proteins is HIF-1α, which gets ubiquitinated upon binding with pVHL and subsequently degraded. However, NR4A1 is able to bind pVHL and prevent this HIF-1α ubiquination [430]. As mentioned, HIF-1α can transcriptionally promote expression of NR4A1, which was shown in VHL-deficient renal cell carcinoma [245], as well as other cancer cell lines [246]. Since both NR4A1 and HIF-1α are known to enhance expression of oncogenic genes via their transactivation functions, it is understandable that they would have a synergistic effect. *VHL* is targeted by miR-21 [312], expression of which would result in increased HIF-1α protein stability and transcriptional activity.

Proteins that phosphorylate NR4A1

Akt. Furthermore, miRNAs may target protein kinases that are responsible for the copious amounts of phosphorylation endured by NR4A1 on its N-terminus [86]. Some of these kinases positively regulate NR4A1 and mediate either its cell death or cell proliferation effects, while other kinases block these functions. Most studies that involve NR4A1-mediated apoptosis use external stimuli to force these effects. For example, gastric cancer cells treated with TPA experience cell death due to the translocation of NR4A1 to the mitochondria and ensuing induction of apoptosis. However, phosphorylation of NR4A1 by Akt, a serine/threonine kinase, blocks the mitochondrial targeting of NR4A1 along with its interaction with Bcl-2 [431]. Other studies have also

verified this negative effect of Akt on NR4A1-induced apoptosis, as well as an inhibitory effect on NR4A1 transcriptional activity [121, 432]. Furthermore, CHD1L indirectly activates Akt, leading to inhibition of cell death [433]. As discussed, CHD1L is also able to block apoptosis by directly binding NR4A1; therefore the activation of Akt is an additional mechanism by which CHD1L can prevent cell death via inhibition of NR4A1. miRNAs that target *AKT1* would theoretically alleviate the suppression of NR4A1 by Akt. Several of these miRNAs act as tumor suppressors, including miR-105 and miR-133b, which were found to act as tumor suppressors in HCC and bladder cancer, respectively, via inhibition of *AKT1* [318, 319].

JNK. On the other hand, phosphorylation of NR4A1 by c-Jun N terminal kinase (JNK) promotes the translocation and mitochondrial targeting of NR4A1. This NR4A1-mediated apoptosis occurs in the presence of several different cytotoxic compounds. Many of these compounds also upregulate NR4A1 expression, including 6-[3-(1-adamantyl)-4-hydroxyphenyl]- 2-naphthalene carboxylic acid (AHPN). This retinoid is a potent inducer of apoptosis in several cancer types, and addition of 3-Cl-AHPC (an analog of AHPN) to lung, prostate, and breast cancer cells induces phosphorylation of NR4A1 by JNK followed by its translocation to the mitochondria and subsequent apoptosis [167, 434]. Treatment of lung cancer cells with 3-Cl-AHPC also inhibits NR4A1 transcriptional activity [167]. In addition, apaensin, a plant-derived natural product, induces apoptosis in lung and breast cancer cells via NR4A1, which is mediated by JNK phosphorylation [435]. Furthermore, glioma and oral squamous cell carcinoma treated with PCH4, a derivative of n-butylidenephthalide, induced apoptosis, which was dependent upon the mitochondrial targeting of NR4A1 mediated by JNK [436, 437]. Interestingly, the nuclear export of NR4A1 occurs in cisplatin-sensitive ovarian cancer cells but not in cisplatin-resistant cells. Cisplatin-induced apoptosis is dependent on the translocation of NR4A1 mediated by JNK phosphorylation [179]. There are no known miRNAs that target human *MAPK8* (JNK), although miR-92a has been shown to target mouse *Mapk8* [323]. Expression of miR-92a would hypothetically suppress NR4A1 translocation and instead allow for cell survival and proliferation via inhibition of JNK.

DNA-PKcs. In addition to JNK, DNA-PKcs also positively regulates NR4A1. DNA-PKcs phosphorylates NR4A1 and increases its protein levels in liver cancer cells. As mentioned previously, this phosphorylation of NR4A1 enhances the transcriptional activity and phosphorylation of p53 by DNA-PK. Hepatoma cells treated with ionizing radiation (IR) leads to DNA-PKcs-mediated upregulation of NR4A1, which results in apoptosis induced by NR4A1 [232]. miR-101 was found to sensitize glioma and lung tumors to radiation via direct suppression of *PRKDC* (encodes DNA-PKcs) [324]. Paradoxically, decreased *PRKDC* by miR-101 should result in reduced NR4A1-mediated apoptosis, but perhaps miR-101-mediated apoptosis is occurring via NR4A1-independent mechanisms.

GSK-3β. As stated earlier, NR4A1 prohibits the transcriptional activity of β-catenin by disrupting the DNA binding of β-catenin and TCF4 and promotes the recruitment of corepressors to Wnt target genes, thereby attenuating tumor growth. However, this negative regulation by NR4A1 is prevented upon phosphorylation by

GSK-3β, which is a phenomenon observed in most clinical colorectal cancers [418]. This phosphorylation of NR4A1 by GSK-3β allows β-catenin to transcriptionally activate genes involved in cell proliferation, leading to cancer progression. This may seem contradictory to the main function of GSK-3β, which is to promote degradation of β-catenin, leading to tumor suppression. However in colorectal cancer, GSK-3β may have evolved in the tumor microenvironment to instead support β-catenin activity via inhibition of NR4A1. Therefore in this scenario, miRNAs that target and inhibit *GSK3B* should lead to decreased tumor growth since GSK-3β can no longer prevent NR4A1-mediated degradation of β-catenin. miR-26a promotes cholangiocarcinoma [325] and miR-346 promotes osteogenic differentiation [326], both of which mediate their effects by directly targeting *GSK3B*.

Target genes of NR4A1

Proliferation and survival genes. Lastly, miRNAs may attenuate the transcriptional activity of NR4A1 by targeting and suppressing the expression of NR4A1 target genes. Transcriptional targets of NR4A1 that mediate its proliferation and survival functions include *CCND2* (cyclin D2) [415], *E2F1* [415, 438], thioredoxin domain containing 5 (*TXNDC5)* [439], and *BIRC5* (survivin) [233, 238]. Many of these genes act as oncogenes, for example survivin is overexpressed in many cancers including pancreatic cancer, where it was found that NR4A1-induced survivin expression is essential for pancreatic cancer cell growth [233, 238]. In addition to survivin, TXNDC5 is also upregulated in several cancer types and was found to promote proliferation and migration while decreasing apoptosis in gastric cancer cells [440]. TXNDC5 is induced by NR4A1 in pancreatic cancer cells, leading to lower stress levels that permit increased cancer cell growth and survival [439]. Cyclin D2 and E2F1 expression induced by NR4A1 was also found to increase cell proliferation [415], although induction of NR4A1 by TPA actually resulted in increased apoptosis via E2F1 in prostate cancer cells [438]. Numerous miRNAs have been found to directly target these genes, and expression of these miRNAs typically results in decreased proliferation and survival (**Table 2-1**).

Pro-inflammatory genes. Furthermore, liver and colon cancer cells treated with bile acids (BAs) experienced a substantial increase in NR4A1 expression, leading to the upregulation of pro-inflammatory genes subsequently identified as NR4A1 target genes. These include *CDK4*, *CCND2*, *BRE*, *RBBP8*, *MAP4K5*, and *STAT5A*, which most likely mediate the increased proliferation and migration observed upon BA-induced NR4A1 overexpression [231]. Surprisingly, NR4A1 was also found to induce the proapoptotic gene *BID* [231]. miRNAs that target and suppress the expression of NR4A1 target genes would therefore attenuate the effects of NR4A1 transcriptional activity, and in this case the miRNAs would act as tumor suppressors in liver and colon cancers (**Table 2-1**).

POMC. An RXR agonist, HX630, suppresses NR4A1 and its transcriptional induction of pro-opiomelanocortin (*POMC*), leading to the inhibition of corticotroph tumor growth [441]. Corticotroph tumors that secrete excessive amounts of adrenocorticotropic hormone (ACTH) can develop into Cushing's disease [441].

Therefore, NR4A1-mediated induction of *POMC* may lead to the formation of corticotroph tumors and downstream Cushing's disease. miRNAs that block expression of POMC would thus decrease the formation of corticotroph tumors and the likelihood of Cushing's disease. Unfortunately, no miRNAs have been verified to target *POMC* as of yet.

Negative regulators of Wnt. Over 70% of melanomas have a mutation in NRAS or BRAF, resulting in aberrant and constitutive signaling of the MAPK pathway and downstream proliferative effects. When *NR4A1* is knocked down in melanoma cells via siRNA, antagonists of the Wnt pathway, DACT1 and CITED1, were upregulated [442]. Therefore, NR4A1 acts to transcriptionally suppress these antagonists, resulting in the continued activation of the Wnt pathway. This further supports the role of NR4A1 as an oncogenic factor, especially in the case of melanoma since NR4A1 has the highest expression in this cancer compared to all other cancer types. Currently, there are no known miRNAs that target *DACT1* and *CITED1*, although several miRNAs are predicted to target *DACT1* in the TargetScan database. Expression of these predicted miRNAs would theoretically support the function of NR4A1 and promote activation of the Wnt pathway.

Myc and Bcl-2. On the other hand, NR4A1 plays a tumor suppressive role in AML by binding to the promoter of *MYC* and suppressing its transcription, thereby preventing its downstream oncogenic effects. NR4A1 was also found to decrease *BCL2* expression, although it is unclear if it directly binds the promoter. Myc and Bcl-2 work together to promote AML, therefore NR4A1-mediated suppression of *BCL2* further attenuates the oncogenic activity of Myc. Furthermore, the leukemogenicity of AML cells was prevented by overexpression of NR4A1 *in vivo* [443]. There are many miRNAs that target *MYC* and *BCL2*, and expression of these miRNAs would support the role of NR4A1 as a tumor suppressor in AML.

Conclusions

The regulatory proteins listed in this chapter are not an exhaustive list of those that regulate and interact with NR4A1. There are many others that target it in different contexts, however only the proteins that have been shown to target and regulate NR4A1 in the context of cancer are discussed. A more thorough review that was recently published includes all interacting factors of NR4A1 and its family members and explores how these interactions affect various physiological processes [444].

In addition to the proteins discussed here, other transcription factors such as Sp1, NF-kB, and CREB have all been shown to bind the promoter of *NR4A1* to modulate its expression [137, 445, 446]. Other binding partners also include NFAT, GATA4, SRC-2, and c-JUN [77, 447-449]. An important binding partner of NR4A1 that mediates the apoptotic effect of NR4A1 is Bcl-2 [123]. Additional kinases such as ERK2, ERK5, RSK2, and p38α also phosphorylate NR4A1 [450-453]. Furthermore, NR4A1 has been shown to bind the promoters and modulate expression of *EDN1* (endothelin 1) and

ITGB4 (integrin beta 4) [448, 454]. However, these proteins were not discussed in detail since the experiments were performed in relatively normal cell lines. Due to the fact that NR4A1 may play different and opposing roles depending on the cell line, it is possible that it may also have different functions in cancer cells compared to normal cells. Because NR4A1 appears to play an oncogenic role in cancer, this chapter discusses the proteins that regulate NR4A1 in a malignant environment.

Many of the miRNAs discussed here correlate well with the role of NR4A1 in cancer. Those miRNAs that are typically overexpressed in cancer and act as oncomiRs appear to support the oncogenic functions of NR4A1 by suppressing proteins that would otherwise attenuate the actions of NR4A1 or repress oncogenic pathways. On the other hand, many of the miRNAs that target genes involved in NR4A1-mediated cancer progression are known tumor suppressors. All of the miRNAs discussed and listed in **Table 2-1** have been verified to target their aforementioned targets using luciferase assays in which the seed region was mutated to confirm direct binding, and most of them were further verified by qPCR and western blot analysis.

It is important to note that in instances where NR4A1 mediates apoptosis in cancer cells, it is being induced to do so upon treatment with cytotoxic compounds. Therefore in the absence of external stimuli, it seems that the normal role of NR4A1 in cancer is to transcriptionally promote expression of genes that are important in cell proliferation and survival.

According to the latest research, there are numerous studies portraying NR4A1 as an oncogenic factor. Glancing at **Table 2-1**, it is apparent that NR4A1 exerts is mitogenic effects via several mechanisms. These include transcriptional regulation by other transcription factors, protein-protein interactions, post-translational modifications such as phosphorylation, and directly binding the promoters of target genes to drive their expression towards a proliferative and pro-survival state. Understanding these regulatory networks is imperative in order to develop more efficacious and personalized treatment for cancer patients.

CHAPTER 3. REGULATION OF NUCLEAR RECEPTOR NR4A1 BY MIR-124*

Introduction

One of the ten hallmarks of cancer is uncontrolled and limitless cell growth. Cells that have acquired the capability to proliferate indefinitely form tumors that may metastasize and develop into cancer. In some cancers, this proliferative signaling is controlled by certain genes that are often overexpressed and thereby constitute ideal therapeutic targets. Some of these target genes include nuclear receptors such as the estrogen receptor in breast cancer or the androgen receptor in prostate cancer. Another nuclear receptor that researchers have identified as a potential therapeutic target is NR4A1.

The nuclear receptor NR4A1 is commonly upregulated in adult cancers and has oncogenic functions. NR4A1 is an immediate-early response gene that acts as a transcription factor to promote proliferation and protect cells from apoptosis. Conversely, NR4A1 can translocate to the mitochondria and induce apoptosis upon treatment with various cytotoxic agents. The roles of NR4A1 in cancers have been investigated mostly in adult cancers, with very few studies in childhood malignancies. NR4A1 is downregulated in leukemia, and *Nr4a1*/*Nr4a3* double-knockout mice quickly develop acute myeloid leukemia before succumbing to the disease [156]; however, the expression and function of NR4A1 have not been well studied in pediatric solid tumors. Recent data from the Pediatric Cancer Genome Project show that NR4A1 is deleted in many hypodiploid acute lymphoblastic leukemia tumors, whereas it is amplified in some patients with Group 4 medulloblastoma (MB) and rhabdomyosarcoma [455].

Because NR4A1 is upregulated in cancer and may have a role in cancer progression, it is of interest to understand the mechanism controlling its expression. MicroRNAs (miRNAs) are responsible for inhibiting translation of their target genes by binding to the 3′UTR and either degrading the mRNA or preventing it from being translated into protein, thereby making these non-coding endogenous RNAs vital regulators of every cellular process. Several miRNAs have been predicted to target *NR4A1*; however, strong evidence showing the regulation of *NR4A1* by any miRNA is lacking. In this study, we used a luciferase reporter assay containing the 3′UTR of *NR4A1* to screen 296 miRNAs and found that miR-124, which is the most abundant miRNA in the brain and has a role in promoting neuronal differentiation, caused the greatest reduction in luciferase activity. Interestingly, we discovered an inverse relationship in Daoy medulloblastoma cells and undifferentiated granule neuron precursors in which *NR4A1* is upregulated and miR-124 is downregulated. Exogenous expression to further

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elevate NR4A1 levels in Daoy cells increased proliferation and viability, but knocking down *NR4A1* via siRNA resulted in the opposite phenotype. Importantly, exogenous expression of miR-124 reduced NR4A1 expression, cell viability, proliferation, and tumor spheroid size in 3D culture. In all, we have discovered miR-124 to be downregulated in instances of medulloblastoma in which NR4A1 is upregulated, resulting in a proliferative state that abets cancer progression. This study provides evidence for increasing miR-124 expression as a potential therapy for cancers with elevated levels of NR4A1.

Methods

Cell culture

Human embryonic kidney cell line 293T (ATCC CRL-3216), human cortical neuronal cell line HCN-2 (ATCC CRL-10742), human medulloblastoma cell lines D341 (ATCC HTB-187) and Daoy (ATCC HTB-186), and human rhabdomyosarcoma cell lines RD (ATCC CCL-136) and SJCRh30 (ATCC CRL-2061) were obtained from ATCC (Manassas, VA). Cells were free from contamination of mycoplasma, and passaged for fewer than 6 months after receipt (or resuscitation). Human rhabdomyosarcoma cell line Rh41 has been described previously [456]. 293T, HCN-2, and RD cells were grown in Dulbecco's Modified Eagle's Medium (DMEM). Rh41 and Rh30 cells were grown in RPMI-1640 Medium, and Daoy cells were cultured in Eagle's Minimum Essential Medium (EMEM). UKF-NB-3 (NB3) cells, which originated from a patient with MYCN-amplified stage 4 neuroblastoma [457], were cultured in Iscove's Modified Dulbecco's medium (IMDM). LHCN-M2 cells (immortalized myoblasts derived from the pectoralis major muscle [458]) were plated in gelatin-coated plates (0.1% gelatin in PBS) and cultured in DMEM supplemented with 15% FBS, 0.02M HEPES, 0.03 μg/mL zinc sulfate, 1.4 μg/mL vitamin B12, 0.055 μg/mL dexamethasone, 2.5ng/mL hepatocyte growth factor (recombinant human), and 10 ng/mL basic fibroblast growth factor. All cells were cultured at 37°C in 5% CO2, and all media (except LHCN-M2 media) were supplemented with 10% fetal bovine serum, 1% GlutaMAX, 1% sodium pyruvate, and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA).

Reporter assay

For the miRNA screen, all miRNA constructs were obtained from an existing library [459]. These constructs are plasmids containing the pre-miRNA sequences and have been reconstituted in TE (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0) buffer. Each miRNA construct (0.09 μg) was first added to the well of a 96-well plate (PerkinElmer, Waltham, MA) that were kept at 4°C until all miRNAs were plated. Next, 0.15 μg of *NR4A1*-3′UTR reporter plasmid (*NR4A1*-3′UTR-Luc; GeneCopoeia, Rockville, MD) and 3 μL/μg of FuGENE 6 (Promega, Madison, WI) were mixed with 50 μL of Opti-MEM reduced-serum media (Life Technologies) and dispensed into each well

before being overlaid with 293T cells (20,000 cells/well in 50 μL of antibiotic-free media). *NR4A1*-3′UTR-Luc (in the pEZX-MT01 vector) contains both the firefly luciferase gene (F*Luc*) fused upstream of the 3′UTR of *NR4A1* under the control of the SV40 promoter and the *Renilla* luciferase gene (R*Luc*) under the control of the CMV promoter. RLuc was used as an internal transfection control. After 48 hours, the Dual-Glo luciferase assay system (Promega) was used to detect luciferase activity according to the manufacturer's instructions. Raw luciferase activity was measured by using the EnVision 2101 Multilabel Plate Reader (PerkinElmer). Raw values were normalized by dividing the F*Luc* values by the RLuc values and then normalized to the value of either pSIF, an empty vector control for miRNA that contains a scrambled sequence in place of the pre-miRNA sequence [459], or oligo control (Cntrl) in the miR-124 inhibitor assay. The F*Luc*/R*Luc* values for pSIF or Cntrl were set as 1. Mutations in the 3′UTR of *NR4A1* that disrupt the binding site of the miRNAs were made by Mutagenex (Suwanee, GA).

RNA isolation and quantitative real-time PCR

Total RNA, including miRNA, was extracted by using the Qiagen miRNeasy kit (Qiagen, Venlo, Netherlands); the Maxwell 16 LEV simplyRNA Tissue Kit was used with the Maxwell 16 Research Instrument (Promega) for RNA extraction only when miRNA extraction was not needed. RNA was converted to cDNA by using the SuperScript VILO cDNA Synthesis Kit (Life Technologies), and 2 μL of 5X Taqman probes (Applied Biosystems) specific to each miRNA were added to enhance miRNA detection. Target gene mRNA expression was detected by using specific Taqman probes (20X) and quantitated via the 7900HT Fast Real-Time PCR System (Applied Biosystems). *GAPDH* (4352934E) was used as an endogenous control for all gene expression analysis, including *NR4A1* (Assay ID Hs00374226_m1), *E2F1* (Assay ID Hs00153451_m1), *CCND2* (Assay ID Hs00153380_m1), *BIRC5* (Assay ID Hs04194392_s1), *TXNDC5* (Assay ID Hs01046709_mH), *CDK4* (Assay ID Hs01565683_g1), and *STAT5A* (Assay ID Hs00234181_m1). Both RNU6B (Assay ID 001093) and RNU48 (Assay ID 001006) were used as endogenous controls for miRNA expression. RNU48 was used instead of RNU6B to analyze endogenous miRNA expression in the cell lines because RNU48 had less variable Ct values among cell lines. The probes used to detect miRNA levels (Applied Biosystems) were miR-124-3p (Assay ID 001182), miR-15a-5p (Assay ID 000389), and miR-224-5p (Assay ID 002099). Gapdh (4352932E) and *Nr4a1* (Assay ID Mm01300401_m1) mouse probes were used to detect *Gapdh* and *Nr4a1* expression in mice. snoRNA202 (Assay ID 001232) was used as an endogenous control for miR-124 expression in mice as recommended by Applied Biosystems [447]. The fold-change in expression was calculated by using the comparative Ct (ΔΔCt) method, with the values of controls set to 1. All samples were tested in quadruplicate. The Cancer miRNAs Transcriptome PCR Array (SA-Biosciences, MD) was used to identify potential miRNAs that target *NR4A1*. The array was provided by the manufacturer in a 96-well PCR plate, each well containing cDNA sample synthesized from HeLa cells treated with one of 90 cancer-related miRNA mimics. According to the manufacturer's instruction, we added qPCR MasterMix and *NR4A1* probe and performed qPCR. We used the data analysis software provided by

SABiosciences to analyze qPCR data and determine which miRNAs affect *NR4A1* expression.

miR-124 inhibitor assay

Daoy cells were plated at a density of 100,000 cells per well in 6-well BD Falcon plates (Corning, Corning, NY). After 24 hours, the cells were first transfected with various concentrations of the miR-124-3p inhibitor (single-stranded RNA molecule) or the control oligonucleotide (oligo) for 24 hours at 37°C and then transfected with 1 μg of the *NR4A1*-3′UTR-Luc reporter plasmid. Transfecting the miR-124-3p inhibitor first allowed it to sufficiently inhibit the miR-124 activity before transfection of the *NR4A1*-3′UTR-Luc reporter plasmid. After 24 hours of incubation, the cells were reseeded at a density of 4,000 cells per well in a 96-well plate, with 6 wells used for each condition. The DualGlo reporter assay was performed 48 hours later. The mirVana inhibitor from Life Technologies and the Power inhibitor from Exiqon (Woburn, MA) were both used to obtain the inhibitor data as indicated in the figure legends; each was used with a control oligo from their respective manufacturers. The Lipofectamine RNAiMAX transfection reagent (Life Technologies) was used with both inhibitors.

Transfections

Cells were transfected with miRNAs by using Fugene6 (Promega) in Opti-MEM in combination with antibiotic-free media (corresponding to the cells being transfected). The plasmid containing pre-miR-124-1 and its backbone vector pEZX-MR03 were purchased from GeneCopoeia.

For the NR4A1 and miR-124 co-transfection assay, Daoy cells were seeded at a density of 100,000 cells per well in a 6-well BD Falcon plate. After 24 hours, cells were transfected with 1 μg NR4A1 with or without its 3′UTR. Cells were transfected with 2.5 μg miR-124 or its control vector (MR03) 24 hours later and collected for Western blot analysis 48 hours after that.

For *NR4A1*-knockdown assays, Daoy cells were seeded at a density of 250,000 cells in T25 flasks. Once the cells were 60%-70% confluent, they were transfected with 20 nM siNR4A1 by using Dharmacon siGENOME siRNA (GE Healthcare, Lafayette, CO) and 8 μL of RNAiMAX. After 48 hours, cells were reseeded for viability and proliferation assays. The SMARTpool siNR4A1 (Catalog # M-003426-04) and the individual siNR4A1 (Catalog # D-003426-23) were both used as indicated in the figure legends. Non-targeting siRNA #4 (Catalog # D-001210-04-20) was used as a control for both the pooled and individual siRNAs.

Molecular cloning

NR4A1 cDNA was cloned into the pEXM12-3XFLAG (N-terminal) vector (GeneCopoeia). Forward (5′– ATACTAGTCCACCATGGACTACAAAGACC –3′) and reverse (5′– ATG AAT TCC TAG AAG GGC AGC GTG TC –3′) primers were used to PCR-amplify 3XFLAG-NR4A1 cDNA from the pEXM12-3XFLAG-NR4A1 vector. The NR4A1 PCR product was then cloned into the pCR2.1 TOPO vector by using the TOPO TA Cloning Kit (Life Technologies). The product was then digested by using SpeI and EcoRI restriction enzymes (New England BioLabs, Ipswich, MA) and ligated into a pSIN-EF2-IRES-Blast lentiviral expression vector to generate pSIN-NR4A1. The pSIN vector originated from Addgene (Plasmid #16578, [460]) but was modified by inserting additional enzyme sites and the *BlastR* gene (for resistance to blasticidin).

To generate a pSIN-*NR4A1*-3′UTR construct, the 3′UTR of *NR4A1* was cloned from the *NR4A1*-3′UTR reporter plasmid (GeneCopoeia) and inserted into the 3′UTR region downstream of the *NR4A1* coding sequence in the pSIN-NR4A1 vector. Briefly, the 3′UTR sequence was amplified by using forward (5′– ATGAATTCCCCCTGCCTGGGAA –3′) and reverse (5′– ATGGATCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCAACTACATGT –3′) primers. This 3′UTR PCR insert was electrophoresed on a gel, and the band was gel-purified by using the Qiagen gel extraction kit. The purified 3′UTR insert was then cloned into the pCR2.1 TOPO vector (Life Technologies). The TOPO and pSIN plasmids were digested by using EcoRI and BamHI (New England BioLabs), and the 3′UTR segment was ligated into the pSIN-NR4A1 vector. All primers were synthesized by Invitrogen; all PCR amplifications were performed by using the Phusion High-Fidelity PCR Master Mix with HF Buffer (New England BioLabs); and the sequences of all final DNA constructs were confirmed by performing Sanger sequencing.

Protein isolation and Western blot analysis

Cells were incubated with Pierce RIPA lysis buffer (Thermo Fisher Scientific, Grand Island, NY) on ice for 20 minutes and centrifuged at 17,500 g for 20 minutes. The supernatant was collected, and its protein concentration was measured by using a Pierce BCA Protein Assay kit. Absorbance at 540 nM was measured by using the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). The protein was mixed with 10X loading buffer and 4X LDS (Life Technologies), incubated at 95°C for 5 minutes, and loaded into a NuPAGE 4-12% Bis-Tris gel (Life Technologies). The separated protein was then transferred to a nitrocellulose membrane by using an iBlot transfer system (Invitrogen). The blot was blocked at room temperature for one hour by using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). Mouse monoclonal anti-Flag M2 (Sigma; catalog # F1804-5MG; used at 1:1500 dilution) and mouse monoclonal anti-β-actin (Sigma; A5441; used at 1:2000 dilution) antibodies were added and incubated overnight at 4°C. After the primary antibodies were removed, the blot was washed three times with TBST for 15 minutes each time before being incubated with the secondary antibody for 1 hour at room temperature. After 1 hour, the blot was washed

three times, and proteins were detected by using the Odyssey imaging system (LI-COR Biosciences). ImageJ [461] was used to measure band intensity.

Lentivirus production and transduction

Two million 293T cells were seeded into each 10-cm dish. Once the cells reached approximately 90% confluence, they were transfected with 12 μg of the expression plasmid, 3 μg of the VSV-G envelope-expressing plasmid pMD2.G (Addgene, Plasmid #12259), and 6 μg of the 2nd-generation lentiviral packaging plasmid psPAX2 (Addgene, Plasmid #12260) by using 60 μL FuGENE6 (Promega) in OptiMEM. Media were replaced with fresh media 24 hours after transfection. The lentivirus supernatant was collected 48 hours after media replacement and filtered through a 0.45-μM filter and titrated by using Lenti-X GoStix (Clontech Laboratories, Mountain View, CA). In cases of low titer, Lenti-X Concentrator (Clontech Laboratories) was used to increase the lentiviral titer.

To exogenously express NR4A1, Daoy cells were seeded at a density of 250,000 cells in T25 flasks. Once the cells were 60%-70% confluent, they were transduced with NR4A1 lentivirus; Polybrene (AmericanBio, Natick, MA) was used at 0.8 μL/mL to aid transduction efficiency. After 48 hours, cells were reseeded for viability and proliferation experiments.

Viability assay

Cells were reseeded at a density of 1000 cells per well in 96-well plates, with 5 replicates of each condition. Cell viability was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega): cells were incubated with 100 μL of the reagent for 20 minutes on a shaker, covered with a dark lid. Luciferase activity was measured by using the EnVision 2101 Multilabel Reader (PerkinElmer) on the day the cells were seeded (day 0) and daily after that for 3-4 days. The day 1 through day 4 viability measurements were normalized to that measured on day 0. The initial seeding density of 1000 cells per well was chosen so that cells would be close to but less than 100% confluent by the final day.

Crystal violet staining

Cells were reseeded in 12-well plates at a concentration of 15,000 cells per well, with 4 replicates of each condition. After removing the media and washing with PBS, we fixed the cells in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO) and gently rocked them for 10 minutes at room temperature. The formaldehyde was then removed, and the cells were washed twice with PBS, incubated with 1% crystal violet (Sigma-Aldrich) while gently rocking for 10 minutes at room temperature, and then rinsed with water until the water washed clear, after which 0.1% SDS was added and incubated for 10 minutes

while gently rocking. The absorbance of each well was then measured at 590 nm by using a SpectraMax M5 (Molecular Devices). Crystal violet staining was performed on the same day as the initial cell seeding (day 0) and daily thereafter for 3-4 days. Crystal violet absorbance readings measured on days 1-4 were normalized to that measured on day 0.

IncuCyte proliferation assays

Cells were reseeded in a 24-well plate at a concentration of 10,000 cells per well, with 4 replicates of each condition. Cell proliferation was monitored by using an IncuCyte live-cell imaging system (Essen BioScience, Ann Arbor, MI): 9 images were captured in each well every 12 hours. The percentage of confluent cells was calculated by using IncuCyte software.

Stable cell lines

Stable cell lines were prepared by plating 500,000 Daoy cells in 10-cm dishes. After 24 hours, cells in antibiotic-free media were transfected with 10 μg of either pEZX-MR03 (vector control) or pEZX-MR03-miR-124 in FuGENE6 diluted in OptiMEM. The media were replaced with normal growth media 24 hours after transfection. The cells were treated with 1 μg/mL puromycin 24 hours after media replacement. Puromycin was added every 3 days for 2 weeks until nontransduced control cells were completely killed by the puromycin, after which the cells were considered to be stable. Expression of miR-124 in the stable cells was confirmed by using a microscope to observe the GFP signal expressed from the vector and by performing qPCR assays to quantify the levels of miR-124.

3D-spheroid formation assay

Parental Daoy cells and Daoy cells stably expressing miR-124 or the control vector (MR03) were seeded into a round-bottom 96-well plate at 3 different densities (288, 800, and 2500 cells/well). Media were changed every 3-4 days, and spheroid areas were calculated after 23 days by using the IN Cell Analyzer 6000 (GE). Viability was also measured on day 23 by using the CellTiter-Glo 3D Cell Viability Assay (Promega) according to the manufacturer's protocol and shown as raw luminescence units (RLU).

Cerebellar granule neuron analysis

Cerebellar granule neurons (CGNs) were prepared as described [462]. Briefly, cerebella were dissected from the brains of P7 C57BL/6 mice, and pial layers were removed; the tissue was treated with trypsin/DNase and triturated into a single-cell suspension by using fine-bore Pasteur pipettes. The suspension was layered onto a

discontinuous Percoll gradient and separated by centrifugation. The small-cell fraction was then isolated. The resulting cultures routinely contained 95% CGNs and 5% glia. The cultures were maintained in Basal Medium Eagle (BME; Life Technologies) supplemented with glutamine and 10% horse serum. All animal experiments were performed in accordance with a protocol approved by St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee. The animals were housed at 22−23ºC with a 12 h light/dark cycle and free access to food and water at the St. Jude Animal Resources Center certified by the American Association for Accreditation of Laboratory Animal Care. Animals were euthanized by decapitation for the preparation of CGNs.

Statistical analysis

Results are shown as the mean ± standard error of the mean. The sample values were compared to control values by using a two-tailed unpaired student's t-test. GraphPad Prism 6 software was used to graph results and to calculate the statistical significance.

Results

Three miRNAs directly target *NR4A1*

To identify miRNAs that may target *NR4A1*, we used a luciferase reporter system in which 293T cells were co-transfected with a reporter plasmid containing the 3′UTR of *NR4A1* along with our collection of 296 miRNAs. In the NR4A1-3′UTR-Luc reporter, the firefly luciferase gene is directly upstream of the 3′UTR sequence: a miRNA that binds to the 3′UTR will decrease the translation of the luciferase mRNA, resulting in decreased luciferase activity being detected by the Dual-Glo Luciferase Assay System. As shown in a waterfall plot (**Figure 3-1A**), miR-124 caused the greatest reduction in luciferase levels among the 296 miRNAs tested. We selected 40 miRNAs that caused 40% or more reduction in luciferase activity and retested them in triplicate: 13 of the 40 miRNAs repeatedly decreased luciferase levels by 30% or more (**Figure 3-1B**).

In addition to using the luciferase reporter system, we used the Cancer miRNAs Transcriptome PCR Array (as described in Materials and Methods), which contains cDNA from HeLa cells transfected with one of 90 cancer-related miRNAs, many of which were included in our collection of 296 miRNAs used in the luciferase reporter screen. We found that miR-124 was one of the 3 miRNAs that substantially downregulated *NR4A1* (**Figure A-1**).

Among the miRNAs that downregulated *NR4A1* (**Figure 3-1A** and **B**), only miR-124, miR-15a, and miR-224 were predicted by multiple prediction algorithms to target *NR4A1* by binding to its seed region (a 5- to 8-nucleotide sequence within the 3′UTR that

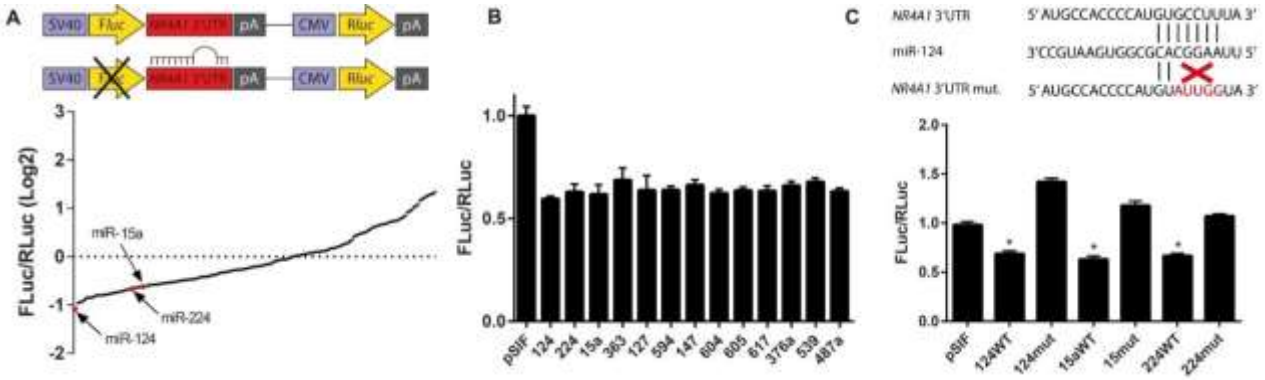

Figure 3-1. miR-124, miR-15a, and miR-224 directly target NR4A1.
(A) miR-124 caused the greatest decrease in luciferase activity after 293T cells were transfected with a NR4A1-3′UTR-Luc reporter and one of 296 miRNAs. Each data point on the waterfall plot corresponds to the resulting luciferase activity for each miRNA. *Renilla* luciferase (RLuc) was used to normalize firefly luciferase (FLuc) activity. **(B)** Thirteen miRNAs significantly ($p < 0.001$) reduced luciferase activity below that of the pSIF control vector (average of 3 independent experiments is shown). **(C)** The seed region where miR-124, miR-15a, and miR-224 are predicted to bind within the 3′UTR of NR4A1 was mutated, and luciferase assays were performed to show direct targeting of NR4A1 by these 3 miRNAs. The data shown are the average of 3 independent experiments. * indicates $p < 0.001$.

mediates the direct binding of a miRNA). These binding predictions are based on the predicted seed regions found within the TargetScan database. When we mutated the binding sites within the 3′UTR corresponding to seed regions for miR-124, miR-15a, and miR-224, the mutated 3′UTR (124mut, 15mut, and 224mut) became resistant to the corresponding miRNA (**Figure 3-1C**), demonstrating that these miRNAs directly target *NR4A1* by binding to a seed region within the *NR4A1* 3′UTR.

NR4A1 is upregulated in pediatric cancer cell lines

To further investigate the functional relationship between NR4A1 and its miRNA regulators, we first analyzed the endogenous *NR4A1* mRNA levels in several pediatric cancer cell lines. These levels were significantly higher in rhabdomyosarcoma cells lines RD, Rh41, and Rh30 than in LHCN-M2 immortalized myoblasts (**Figure 3-2A**). In addition, *NR4A1* expression in D341 and Daoy medulloblastoma cells and in NB3 neuroblastoma cells was upregulated compared to that of HCN-2 human cortical neurons (**Figure 3-2B**). We further analyzed the endogenous expression of miR-124, miR-15a, and miR-224 in Rh30, Daoy, and NB3 cells. As shown in Fig 2C, all 3 miRNAs were downregulated in Daoy, and miR-224 was decreased in NB3 cells. Interestingly, miR-124 was upregulated in NB3, a *MYCN*-amplified neuroblastoma cell line [457], which is consistent with a recent report showing miR-124 upregulation in MYCN-amplified neuroblastoma when compared to nine other pediatric solid tumors including rhabdomyosarcoma and non-*MYCN*-amplified neuroblastoma [463]. The mechanism responsible for the upregulation of miR-124 in NB3 cells is unclear. miR-224 was significantly downregulated in NB3 cells (**Figure 3-2C**), possibly contributing to the upregulation of *NR4A1* (**Figure 3-2B**). For the remainder of this study, we focused on the relationship between miR-124 and NR4A1 in Daoy cells because miR-124 is highly expressed in the brain [464] and it acts as a tumor suppressor in medulloblastoma [465-467]. Furthermore, in undifferentiated granule neuron precursors (GNPs), the level of *Nr4a1* was high but that of miR-124 was low (**Figure A-2**). Interestingly, once these GNPs differentiated, *Nr4a1* was downregulated and miR-124 was upregulated (**Figure A-2**). These observations led us to further investigate the functional relationship between miR-124 and NR4A1.

miR-124 decreases NR4A1 expression

Compared to expression in HCN-2 cells, *NR4A1* was upregulated and miR-124 was downregulated in Daoy cells (**Figure 3-3A**). We further investigated the inverse correlation between NR4A1 and miR-124 expression by determining the effect of modulating miR-124 levels on the levels of *NR4A1*. As shown in Fig 3B, higher levels of miR-124 correlated with lower levels of *NR4A1*. Additionally, the NR4A1 3′UTR-Luc activity increased after treatment with a miR-124 inhibitor, further validating the inverse relationship between miR-124 and NR4A1 (**Figure 3-3C**). Another miR-124 inhibitor (from Exiqon) was used, yielding similar results (**Figure A-3**). Exogenous overexpression of miR-124 decreased the mRNA level of endogenous NR4A1 and that of

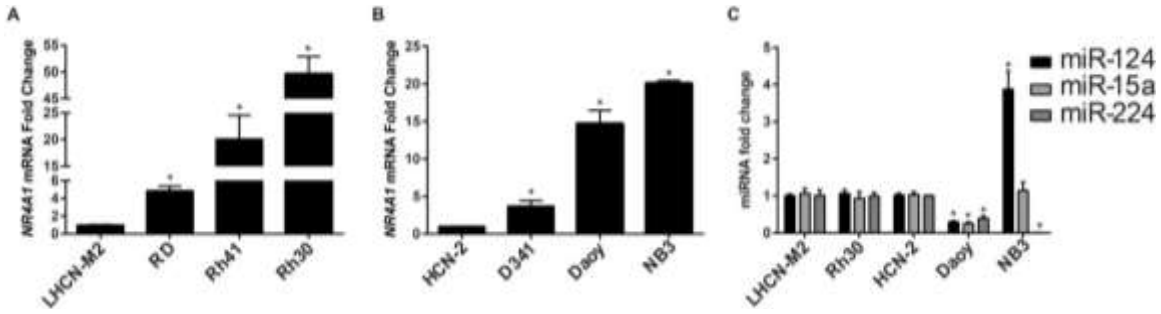

Figure 3-2. *NR4A1* is upregulated in pediatric cancer cell lines.
(A, B) *NR4A1* mRNA expression is upregulated in rhabdomyosarcoma, medulloblastoma, and neuroblastoma cell lines. Fold-change was calculated by normalizing the mRNA expression levels to those of their respective control cell lines (either LHCN-M2 or HCN-2), which were set to 1. **(C)** Endogenous miRNA expression in Rh30, Daoy, and NB3 cells shows that all 3 miRNAs are downregulated in Daoy cells and that miR-224 is downregulated in NB3 cells. RNU48 was used as an internal control. All data shown are the average of 3 independent experiments; $*p < 0.0001$.

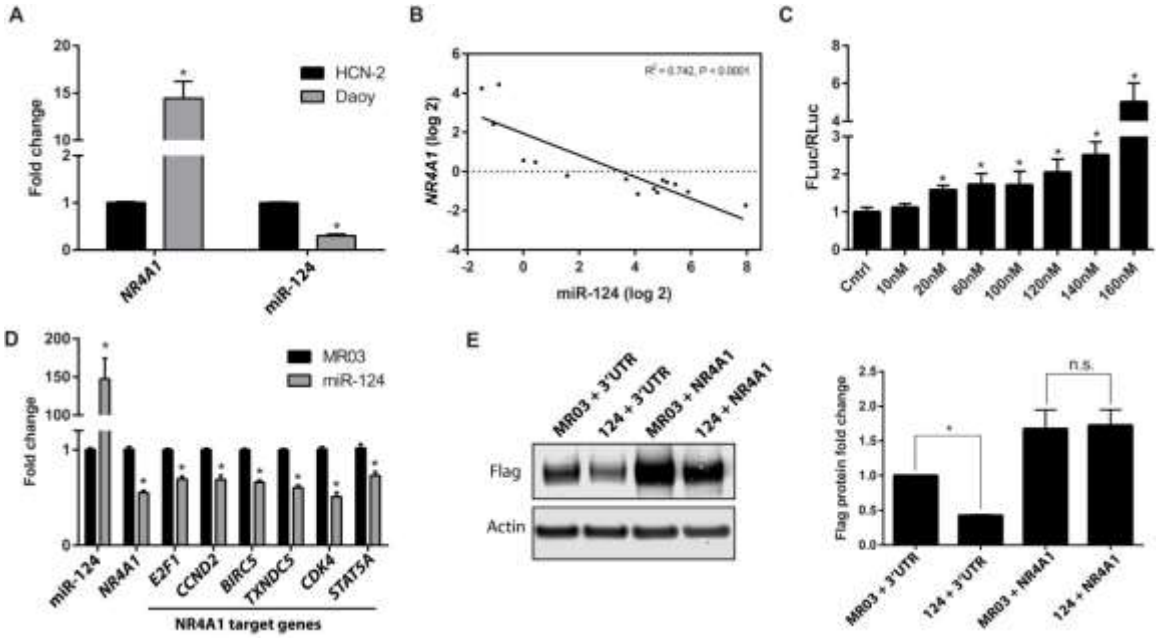

Figure 3-3. miR-124 decreases *NR4A1* levels.
(A) Endogenous expression levels of miR-124 and *NR4A1* were measured in Daoy cells and human cortical neurons (HCN-2). **(B)** *NR4A1* and miR-124 expression are inversely related in Daoy cells exogenously expressing various levels of miR-124. The levels of *NR4A1* and miR-124 changed in an inversely correlated manner. **(C)** Daoy cells were co-transfected with the NR4A1-3′UTR reporter plasmid (NR4A1-3′UTR-Luc) and either an inhibitor of miR-124 (10 – 160 nM of oligonucleotide used as indicated) or an oligo control (Cntrl) from Life Technologies; resulting luciferase levels were measured. The data shown are representative of 3 independent experiments. **(D)** Either miR-124 or the control vector (MR03) was exogenously expressed in Daoy cells, and the resulting levels of miR-124 and *NR4A1* were measured along with the expression of NR4A1 target genes. **(E)** Daoy cells were co-transfected with miR-124 (124) or vector control (MR03) and Flag-NR4A1 plasmid with (3′UTR) or without (NR4A1) the 3′UTR to confirm that miR-124 targets the 3′UTR. Flag and actin protein levels were detected by Western blot and quantified by using ImageJ. Levels of Flag protein were first normalized to those of actin; then MR03-3UTR was set to 1, and all other samples were compared to this sample. The Western blot shown is representative of 3 independent experiments, and the bar graph shows the average protein fold-change from 3 experiments. * indicates $p < 0.05$.

several target genes of NR4A1, including *E2F1*, *CCND2* (cyclin D2), *BIRC5* (survivin), *TXNDC5*, *CDK4*, and *STAT5A* (**Figure 3-3D**). Furthermore, miR-124 overexpression also decreased the expression of NR4A1 target genes in 293T cells (**Figure A-4**). To demonstrate that elevated miR-124 decreases NR4A1 protein levels in a 3′UTR-dependent manner we examined the effect of overexpressed miR-124 on a Flag-tagged NR4A1 construct without the 3′UTR (NR4A1) or with the 3′UTR (3′UTR). As shown in **Figure 3-3E**, overexpression of miR-124 decreased the level of Flag-NR4A1 only when the 3′UTR of NR4A1 was present.

NR4A1 promotes cell viability and proliferation

Studies have shown that NR4A1 increases cell survival and proliferation in various adult cancer cell lines, suggesting an oncogenic role for NR4A1 in those particular cancers [230, 233, 239, 468-470]. We examined whether this was also the case for pediatric cancer cell lines, specifically Daoy medulloblastoma cells. Upon exogenous overexpression of NR4A1 in Daoy cells, there was increased cell viability as assessed by the CellTiter-Glo assay (**Figure 3-4A**). Cell proliferation was measured by performing crystal violet staining and using an IncuCyte live-cell imaging system, which monitors real-time cell proliferation. Compared to cells transduced with the empty vector (EV), the cells exogenously overexpressing NR4A1 showed increased crystal violet staining and confluence over the course of 4 days (**Figure 3-4B** and **C**). **Figure 3-4D** shows the elevated *NR4A1* mRNA levels after transduction with NR4A1. These data support the notion that NR4A1 promotes tumor growth not only in adult cancers but also in pediatric solid tumors.

Knockdown of *NR4A1* decreases cell viability and proliferation

To further validate the effects of NR4A1 on cell proliferation and viability, *NR4A1* was knocked down via pooled siRNA targeting *NR4A1* (siNR4A1). Daoy cells transfected with siNR4A1 exhibited decreased cell viability and proliferation as measured by the CellTiter-Glo assay, crystal violet staining, and the IncuCyte assay (**Figure 3-5A-C** and **E**). **Figure 3-5D** shows the knockdown efficiency. The 4 individual siRNAs making up the pooled siRNA were then tested individually. siNR4A1_4 most efficiently knocked down *NR4A1* and caused the greatest decrease in cell viability and proliferation (**Figure A-5**).

miR-124 decreases cell viability and proliferation

The observations that NR4A1 promoted cell proliferation (**Figures 3-4** and **3-5**) and that miR-124 directly targeted and downregulated NR4A1 (**Figure 3-3**) led us to examine the effects of miR-124 on cell viability and proliferation. Exogenous and stable expression of miR-124 in Daoy cells substantially reduced cell viability and proliferation (**Figure 3-6A-C**). To measure the ability of the Daoy cells to form microtumors, we used

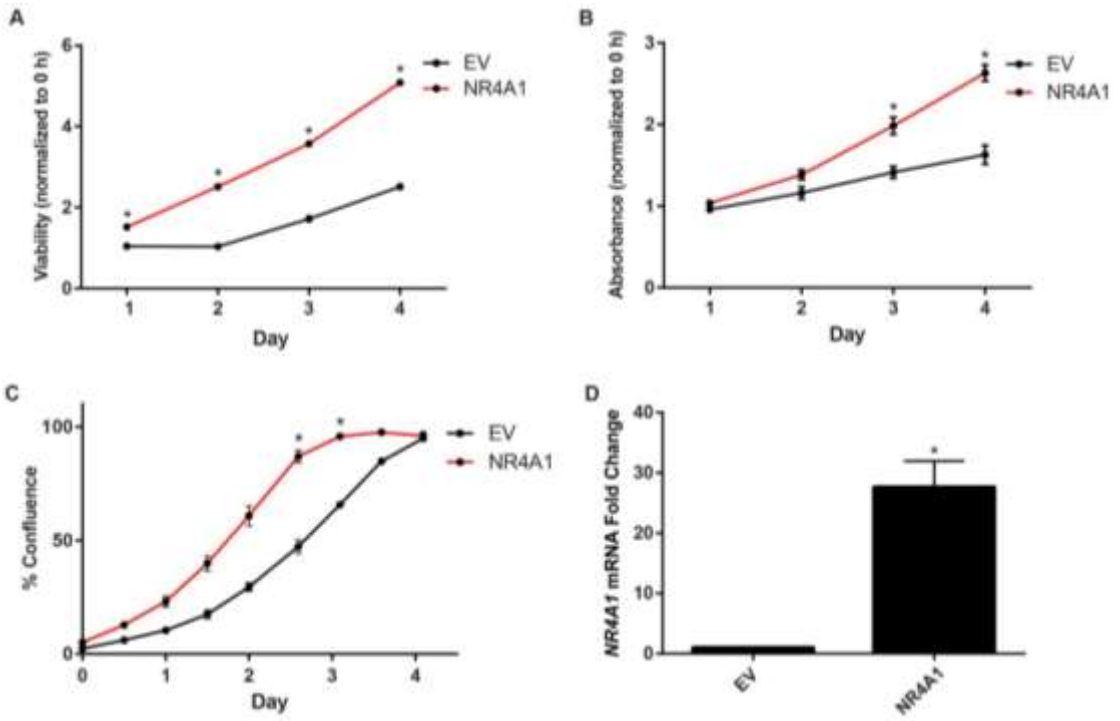

Figure 3-4. NR4A1 promotes cell viability and proliferation.
(A) Daoy cells were transduced with pSIN-NR4A1 (NR4A1) or pSIN vector (EV), and cell viability was measured via the CellTiter-Glo assay every day for 4 days. Viability for each day was normalized to that of Day 0 (0 hours), and statistical significance was calculated for each day. **(B)** Cells were stained with crystal violet every day for 4 days to measure proliferation over time. The absorbance was measured and normalized to that of Day 0 (0 hours). The statistical significance was calculated for each day. **(C)** Cell proliferation was monitored by using an IncuCyte live-cell imager for real-time imaging. The resulting cell confluence was recorded every 12 hours for 4 days. **(D)** *NR4A1* mRNA level was measured after transduction with NR4A1. All experiments were performed by using Daoy cells transduced with EV or NR4A1 lentivirus. All data shown are representative of 3 independent experiments; $*p \leq 0.0001$.

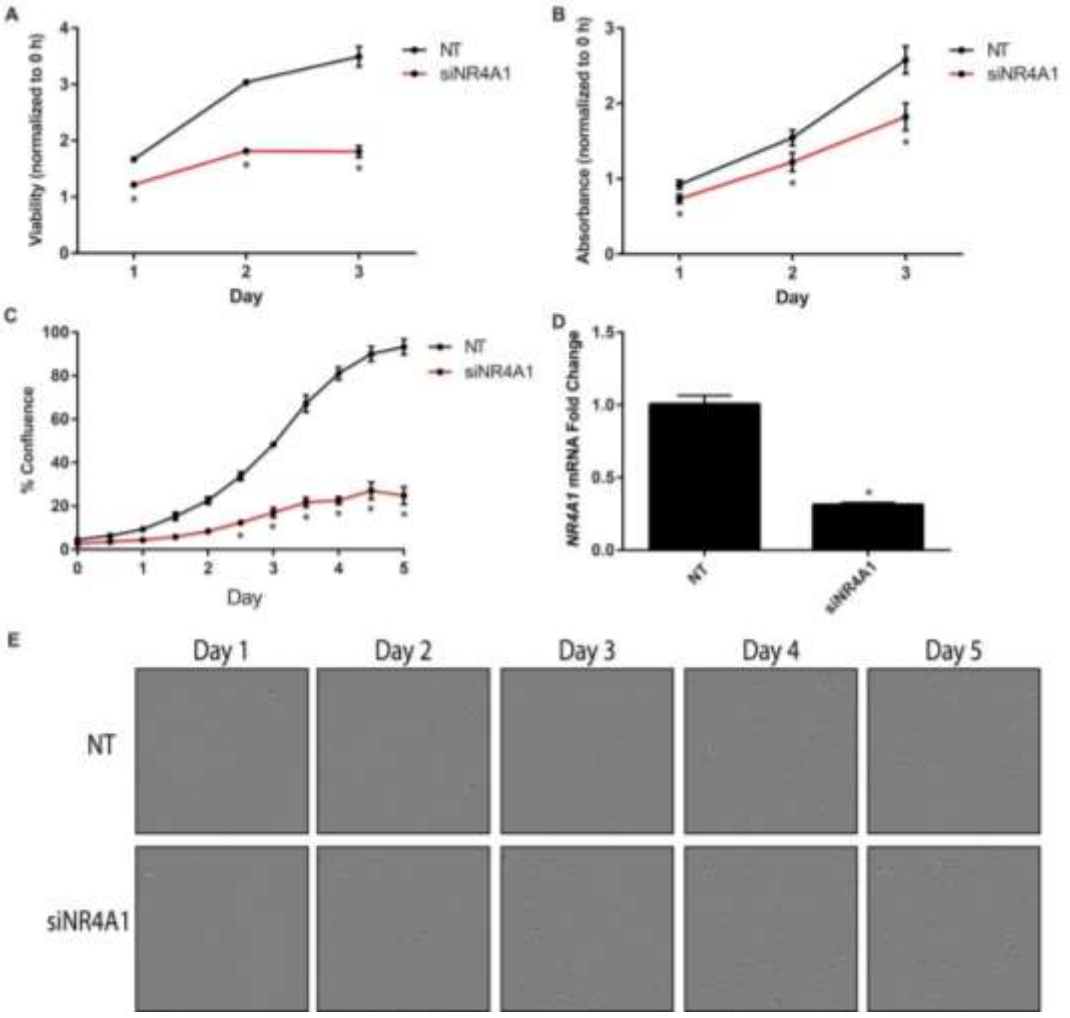

Figure 3-5. ***NR4A1*** **knockdown decreases cell viability and proliferation.** **(A)** Daoy cells were transfected with 20 nM of the SMARTpool siNR4A1 or non-targeting control (NT), and cell viability was measured via the CellTiter-Glo assay every day for 3 days. Viability for each day was normalized to that of Day 0 (0 hours), and statistical significance was calculated for each day; $*p < 0.0001$. **(B)** Cells were stained with crystal violet every day for 3 days to measure proliferation over time. The absorbance was measured and normalized to that of Day 0 (0 hours). The statistical significance was calculated for each day; $*p < 0.01$. **(C)** Proliferation was monitored via the IncuCyte live-cell imager. Cell confluence was averaged, with 4 replicates of each condition; $*p < 0.0001$. **(D)** *NR4A1* was significantly ($p < 0.0001$) decreased after transfecting Daoy cells with siNR4A1. **(E)** Images shown for each NT and siNR4A1 panel over 5 days are the same image view within the same well and are representative of 3 independent experiments with 4 wells for each condition. These images correspond to the data in C. Data shown in A are representative of 5 independent experiments; data in B are representative of 4 independent experiments, and data in C and E are representative of 2 independent experiments. Data shown in D is the average of 4 independent experiments.

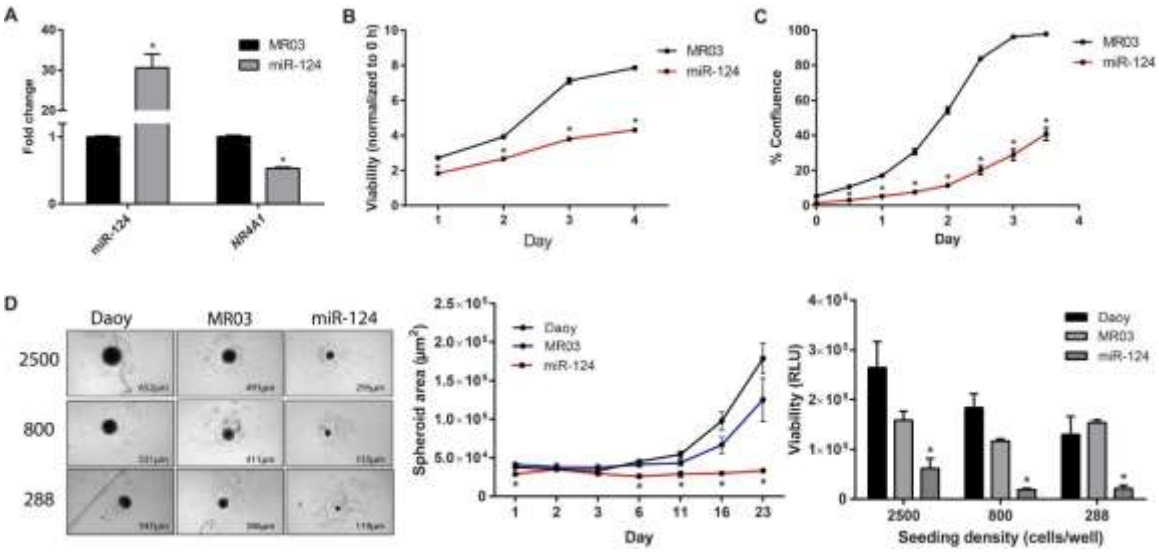

Figure 3-6. miR-124 decreases cell proliferation in 2D and 3D cultures.
(A) Expression of miR-124 was significantly ($p < 0.0001$) increased after antibiotic selection of Daoy cells transduced with pEZX-MR03-miR-124. As a result, *NR4A1* mRNA levels were significantly decreased ($p < 0.0001$). Data shown are the average of 6 independent experiments. **(B)** The CellTiter-Glo assay was used to analyze the cell viability of Daoy cells stably expressing exogenous miR-124 or vector control (MR03). Viability for each day was normalized to that of Day 0 (0 hours), and statistical significance was calculated for each day; $*p < 0.0001$. **(C)** Stable cells were imaged by using the IncuCyte live-cell imager to determine cell proliferation over the course of 3.5 days, and statistical significance was determined for each day; $*p < 0.0001$. **(D)** Parental Daoy cells (Daoy) and Daoy cells stably expressing exogenous miR-124 (miR-124) or its control vector (MR03) were seeded at 3 densities (288, 800, and 2500 cells/well) and grown using 3D culture techniques. After 23 days (left panel), the cells' spheroid areas ($*p < 0.01$) were measured by using the IN Cell Analyzer (middle panel). Viability ($*p < 0.05$) was determined by performing CellTiter-Glo 3D Cell Viability Assays and is shown as raw luminescence units (RLU) (right panel). The spheroid area data shown are for cells seeded at an initial density of 800 cells per well. Data from B are representative of 5 independent experiments; data from C are representative of 4 independent experiments, and data from D are representative of 2 independent experiments.

round-bottom 96-well plates to promote the formation of 3D spheroids. After allowing the cells to form spheroids for 23 days, we found that cells exogenously overexpressing miR-124 underwent less spheroid growth and viability than did the control cells (**Figure 3-6D**). These results are consistent with previously reported evidence showing the negative effects of miR-124 on medulloblastoma cell growth [465-467].

Conclusions

To our knowledge, our study is the first to show that the level of NR4A1 is regulated by miR-124 and that NR4A1 has roles in proliferation of pediatric cancer cells such as Daoy medulloblastoma cells. Most published research on NR4A1 as a therapeutic target involves using drugs to induce NR4A1-mediated apoptosis; our discovery that miR-124 regulates NR4A1 suggests that it may be possible to modulate NR4A1 and influence cancer cell growth by regulating miR-124.

In a panel of pediatric cancer cell lines, we found *NR4A1* to be upregulated in all cell types including RD, Rh41, and Rh30 RMS cells, D341 and Daoy medulloblastoma cells, and NB3 neuroblastoma cells. We identified 3 miRNAs that directly target *NR4A1*, including miR-124, miR-15a, and miR-224, all of which were downregulated in Daoy, demonstrating an inverse correlation between NR4A1 and the miRNAs that target it. However, we did not observe this trend in the other cell types. There are most likely additional miRNAs that target *NR4A1* in the other cell types and perhaps this is why we did not see a correlation.

It is difficult to conclusively determine the role of NR4A1 in medulloblastoma based on these studies as Daoy cells are not classified as any of the 4 subtypes of medulloblastoma. Furthermore, there is not substantial evidence in expression databases demonstrating whether or not NR4A1 is upregulated in patients with medulloblastoma. Therefore, further studies showing expression levels of NR4A1 in medulloblastoma are required in order to make this determination. Furthermore, tumorigenicity assays such as soft agar colony formation assays that test anchorage independent growth can be performed in multiple medulloblastoma cell types that more closely resemble the different subtypes of medulloblastoma. The tumorigenic potential of NR4A1 can also be tested *in vivo* wherein NR4A1 can be overexpressed in a medulloblastoma cell line that is then injected into mice and the resulting tumors are measured to determine if NR4A1 promotes the growth of medulloblastoma tumors. Additionally, knockdown studies can be conducted *in vivo* to conclusively confirm whether or not NR4A1 affects the growth rate of medulloblastoma tumors.

Interestingly, we found that *NR4A1* expression decreases while miR-124 expression increases in differentiating GNPs. This supports previous research showing that miR-124 expression is higher in differentiated GNPs compared to undifferentiated GNPs. The decrease in *NR4A1* expression suggests that NR4A1 must be downregulated, possibly by miR-124, in order for differentiation to occur. It is of interest to explore the function of NR4A1 in GNPs as these are the main type of neuron in the cerebellum where

a subtype of medulloblastoma originates. A more thorough discussion on this topic can be found in Chapter 5.

In short, we found that miR-124 targets and decreases NR4A1 expression and function. NR4A1 promotes cell proliferation in Daoy medulloblastoma cells, but miR-124 reduces it, in part by targeting *NR4A1* and decreasing its transcriptional activity. This results in decreased expression of NR4A1 target genes important in cell cycle progression and survival. This study supports the use of miRNA mimics to treat cancers, especially those in which NR4A1 has an oncogenic role.

CHAPTER 4. THE ROLE OF NR4A1 IN SKELETAL MUSCLE DIFFERENTIATION

Introduction

Diseases related to muscle such as Duchenne muscular dystrophy and rhabdomyosarcoma, which are prevalent in children, are in desperate need of effective treatments. In order to develop therapies, it is critical to understand normal muscle development and function. The process of muscle formation, termed myogenesis, involves several stages including proliferation, migration, and differentiation. During the first stage, mesodermal progenitors exit the cell cycle and several transcription factors appear including Pax3, Pax7, Myf5, and MyoD1. These transcription factors are required for the cells to become myoblasts. These myoblasts will then align with one another and become myocytes after the expression of additional transcription factors including myogenin (MYOG) and Myf6. During the final stages of differentiation, the myocytes will fuse to form multinucleated myotubes, which will then become muscle fibers. Additional muscle markers such as myosin heavy chain (MHC/MYH) and muscle creatine kinase (CKM) become expressed in the later stages of differentiation [471-473].

NR4A1 has many physiological roles in various tissues such as its involvement in muscle function. For example, Nr4a1 promotes glucose and oxidative metabolism in skeletal muscle and has even been shown to promote myofiber size and muscle mass in mice [148, 474, 475]. However, the role of NR4A1 during human skeletal muscle differentiation is not well understood. For these studies, we utilized primary SkMC and HSMM as well as immortalized LHCN human skeletal muscle cells. In this study we found that *NR4A1* expression increases during the myogenic program in LHCN, and knockdown of *NR4A1* results in decreased expression of myogenic markers in all 3 cell types. This suggests that NR4A1 participates in skeletal muscle differentiation.

In addition, ZNF148 may regulate *NR4A1* during myogenesis by suppressing its expression. Knockdown of *ZNF148* in LHCN rapidly induced the formation of myotubes with a resulting increase in *NR4A1* expression. Understanding these mechanisms may be beneficial in cancer since certain malignancies such as rhabdomyosarcoma result from the failure of cells to differentiate and instead proliferate uncontrollably, leading to tumor formation and cancer progression.

Methods

Cell culture

LHCN-M2 (LHCN) cells are human skeletal myoblasts [458] and were grown in flasks coated with extracellular matrix (0.2% MaxGel ECM in PBS from Sigma-Aldrich) and cultured in DMEM supplemented with 15% FBS, 0.02M HEPES, 0.03 µg/mL zinc

sulfate, 1.4 µg/mL vitamin B12, 0.055 µg/mL dexamethasone, 2.5ng/mL hepatocyte growth factor (recombinant human), 10 ng/mL basic fibroblast growth factor, and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA). Differentiation was induced by the addition of differentiation media, which consisted of DMEM supplemented with 0.02M HEPES, 0.03 µg/mL zinc sulfate, 1.4 µg/mL vitamin B12, 10 µg/mL insulin, 100 µg/mL apo-transferrin, and 1% penicillin-streptomycin. Human skeletal muscle cells (SkMC) and myoblasts (HSMM) were purchased from Lonza (Walkersville, MD) and originate from gestational tissue and adult tissue, respectively. They were cultured in SkGM-2 Skeletal Muscle Cell Growth Medium-2 (BulletKit from Lonza) and were differentiated by adding 2% horse serum to DMEM/F-12 media (Lonza). All cells were cultured at 37°C in 5% CO2.

Transfection

LHCN were plated at 100k cells/well in 6-well plates coated with ECM. After 24 hours they were transfected with 10 nM non-targeting control (NT) or siRNA for *NR4A1* (siNR4A1) and 5 ul RNAiMAX (in 300 ul of OptiMEM). Media was replaced with fresh media 24 hours after transfection. Differentiation media was added once the cells reached 100% confluence, which was typically 72 hours post-transfection. Media was replaced every 2 days during the course of differentiation. For knockdown of ZNF148, cells were transfected with 10 nm control (Cntrl) or siZNF148 (Cat. # D-012658-04). Reagents for siRNA were purchased from Dharmacon using their siGENOME siRNA (GE Healthcare, Lafayette, CO). For both siNR4A1 and siZNF148, the knockdown efficiency was approximately 90%, and 3 out of 4 of the individual siRNAs had a similar phenotype, indicating that they are specific to their respective target genes. SkMC and HSMM were plated at 150k cells/well in 6-well plates and were transfected 48 hours after plating. Once cells reached 70-80% confluence, differentiation was induced by the addition of differentiation media, which was replaced every 2 days.

RNA extraction and qPCR

RNA was extracted by using the Maxwell 16 LEV simplyRNA Tissue Kit in conjunction with the Maxwell 16 Research Instrument (Promega). The resulting RNA concentration was measured by using the NanoDrop and was subsequently converted to cDNA by using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). Target gene mRNA expression was detected by using specific Taqman probes (20X) and quantitated via the 7900HT Fast Real-Time PCR System (Applied Biosystems). *18S* (Assay ID Hs99999901_s1) was used as an endogenous control for all gene expression analysis, including *NR4A1* (Assay ID Hs00374226_m1), *MYOG* (Assay ID Hs01072232_m1), *MYH2* (Assay ID Hs00430042_m1), *CKM* (Assay ID Hs00176490_m1), and *ZNF148* (Assay ID Hs01070570_m1).

Protein isolation and Western blot analysis

Cell pellets were lysed in Pierce RIPA lysis buffer (Thermo Fisher Scientific, Grand Island, NY) and incubated on ice for 30 minutes prior to sonication for 5 s and finally centrifugation at 17,500 g for 5 minutes. The protein concentration was measured by using a Pierce BCA Protein Assay kit and absorbance was measured at 540 nM by using the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). The protein lysate was mixed with 10X loading buffer and 4X LDS (Life Technologies) and incubated at 95°C for 5 minutes. Subsequently, the protein mixture was loaded into a NuPAGE 4-12% Bis-Tris gel (Life Technologies) and run at 100 V for 2 hours. The protein was then transferred to a PDVF membrane using the slow transfer method. This method involves activation of the PDVF membrane with methanol for 10 minutes followed by incubation in transfer buffer to remove the methanol. The protein was transferred to the PDVF membrane overnight on ice at 0.45 amperes. The blot was then blocked at room temperature for one hour by using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). Mouse monoclonal anti-MHC (produced using hybridoma cells from the Developmental Studies Hybridoma Bank; used at 1:200 dilution), mouse monoclonal anti-myogenin (Santa Cruz; used at 1:500), and mouse monoclonal anti-β-actin (Sigma; A5441; used at 1:2000 dilution) antibodies were added and incubated overnight at 4°C. After the primary antibodies were removed, the blot was washed with TBST three times for 15 minutes each time prior to incubation with the secondary antibody (LI-COR Biosciences; used at 1:10,000 dilution) for 1 hour at room temperature. Subsequently, the blot was washed three times with TBST and proteins were detected by using the Odyssey imaging system (LI-COR Biosciences).

Immunofluorescence

Cells were washed 3 times with PBS and fixed with 2% paraformaldehyde for 15 minutes at room temperature followed by additional washes with PBS. The cells were then permeabilized with 0.1% Triton X-100 for 15 minutes followed by 3 washes with PBS. The monolayer was then washed 5 times with 0.5% BSA diluted with PBS (PBB) and blocked with 2% BSA for 45 minutes followed by 5 washes with PBB. The primary antibody for myosin heavy chain 1 (MHC) was added at a 1:100 dilution overnight at 4°C and then washed 5 times with PBB. The secondary antibody was used at a 1:500 dilution and incubated for 1 hour at room temperature followed by 5 washes with PBB. DAPI was added at a 30,000 dilution of 1mg/ml for 5 minutes followed by 5 washes with cold PBS. The chambers were then removed and mounted with coverslips and mounting media and imaged using a Nikon Eclipse Ti microscope.

Statistical analysis

Results are shown as the mean ± standard error of the mean. Significance was determined by using a two-tailed unpaired student's t-test to compare the sample values

to the control values. GraphPad Prism 6 software was used to graph results and to calculate the statistical significance.

Results

NR4A1 expression increases during LHCN myogenesis

To determine if NR4A1 plays a role during myogenesis, expression was analyzed during the differentiation of LHCN, SkMC, and HSMM human skeletal muscle cells. *NR4A1* mRNA levels increased by about 4 fold during the differentiation of LHCN, however it did not substantially increase in SkMC and HSMM cells. To verify that the cells were indeed differentiated, the myogenic markers MYOG, MYH2, and CKM were measured (**Figures 4-1**, **4-2**, and **4-3**). MYOG is considered an early marker whereas MYH2 and CKM are late differentiation markers. In order to determine if the other NR4A family members are playing a role, the mRNA levels of *NR4A2* and *NR4A3* were measured in all 3 cell types. *NR4A2* was greatly decreased in LHCN and SkMC but not in HSMM, whereas *NR4A3* was increased in all 3 cell types. This suggests that NR4A2 may inhibit differentiation while NR4A3 may promote it.

NR4A1 knockdown delays differentiation

To determine the significance of the increased *NR4A1* expression in LHCN, *NR4A1*was knocked down via siRNA (siNR4A1). As a result, differentiation was severely delayed as shown by the decrease in differentiation markers and the visible lack of myotube formation (**Figure 4-4B-D**). Protein levels for MYOG and MHC were also extremely decreased in the cells lacking *NR4A1* (**Figure 4-4E**). Bright-field images of the cells clearly show well-differentiated NT cells by Day 4 compared to the siNR4A1 cells that did not form myotubes (**Figure 4-4F**). Interestingly, *NR4A1* expression was induced up to 15 fold by Day 5 in the cells transfected with the non-targeting (NT) control (**Figure 4-4A**). This is a much larger induction compared to the untransfected LHCN in **Figure 4-1A**. Similar results were observed in SkMC and HSMM, however the *NR4A1* mRNA levels actually decreased during differentiation in the NT cells (**Figures 4-5** and **4-6**).

ZNF148 knockdown induces differentiation

Studies from our lab have demonstrated that knockdown of the transcription factor *ZNF148* rapidly induces differentiation in LHCN cells, suggesting that ZNF148 plays an inhibitory role during myogenesis. One study has shown that ZNF148 can suppress the expression of *NR4A1* by directly binding to its promoter [446]. To determine the functional significance of this inhibitory mechanism, *ZNF148* was knocked down via siRNA in LHCN cells which resulted in the rapid formation of myotubes by Day 2

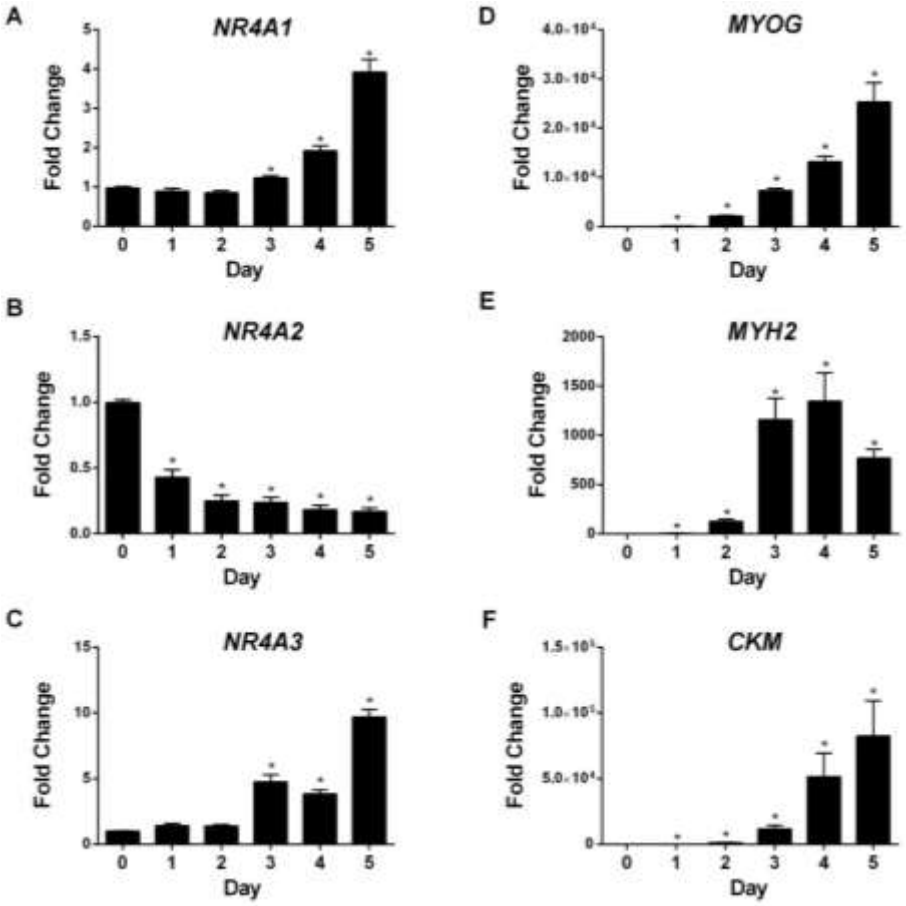

Figure 4-1. *NR4A1* expression increases during LHCN differentiation.
(A-F) LHCN were differentiated for 5 days with cells collected for each day beginning on Day 0 when differentiation media is added. The mRNA levels for *NR4A1*, *NR4A2*, *NR4A3*, myogenin (*MYOG*), myosin heavy chain (*MYH2*), and muscle creatine kinase (*CKM*) were measured and normalized to Day 0, which was set to 1. Data shown is the average of at least 2 independent experiments. * indicates $p < 0.01$.

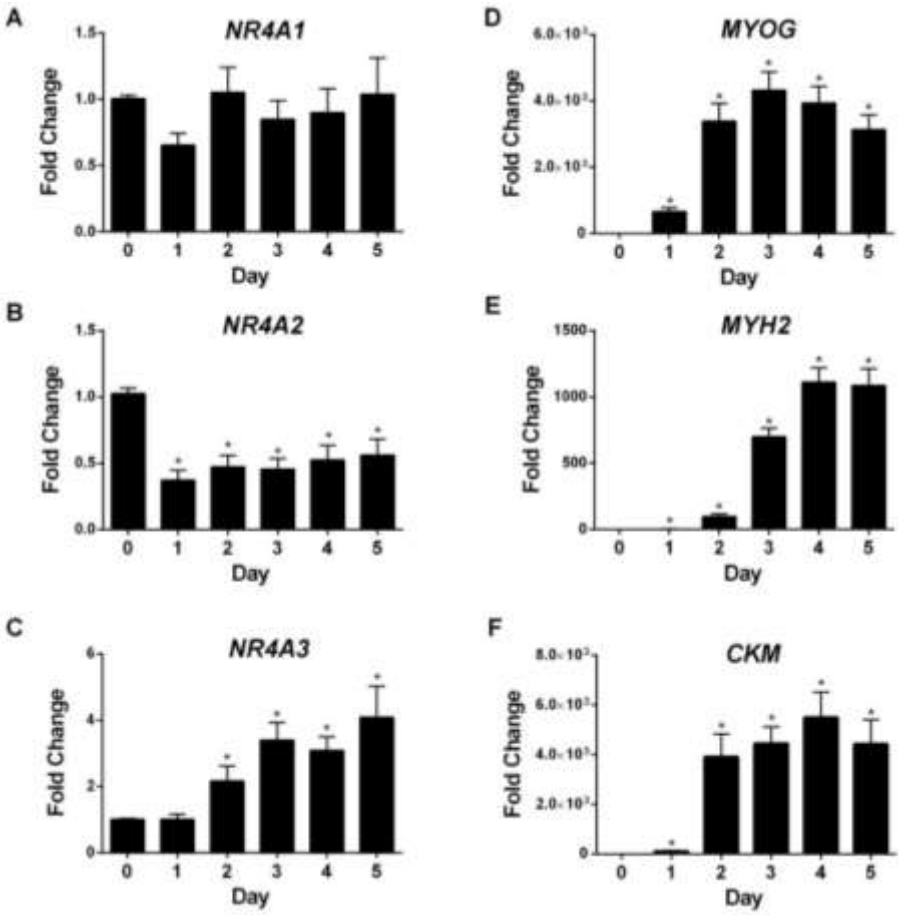

Figure 4-2. ***NR4A1*** **expression does not increase during SkMC differentiation.** **(A-F)** SkMC were differentiated for 5 days with cells collected every day beginning on Day 0 when differentiation media was first added. The mRNA levels of *NR4A1*, *NR4A2*, *NR4A3*, *MYOG*, *MYH2*, and *CKM* were measured and normalized to Day 0, which was set to 1. Data shown is the average of 5 independent experiments. * indicates $p < 0.05$.

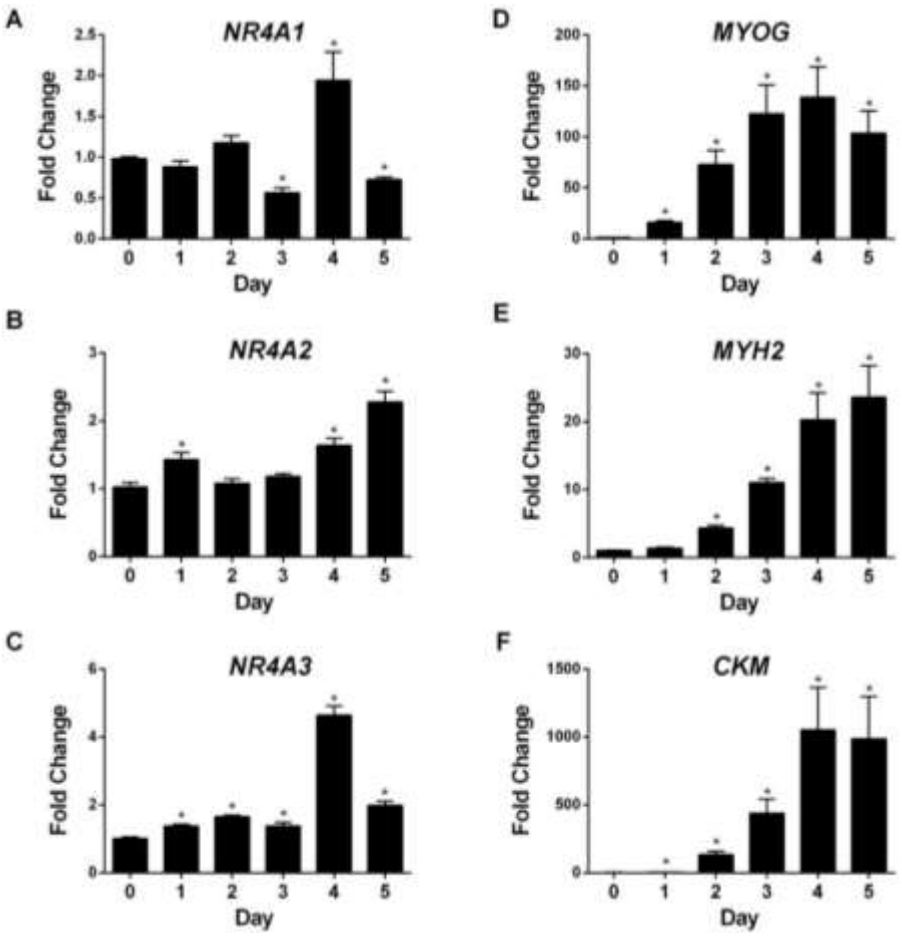

Figure 4-3. *NR4A1* expression does not increase during HSMM differentiation. **(A-F)** HSMM were differentiated for 5 days with cells collected every day beginning on Day 0 when differentiation media was first added. The mRNA levels of *NR4A1*, *NR4A2*, *NR4A3*, *MYOG*, *MYH2*, and *CKM* were measured and normalized to Day 0, which was set to 1. Data shown is the average of at least 2 independent experiments. * indicates $p < 0.05$.

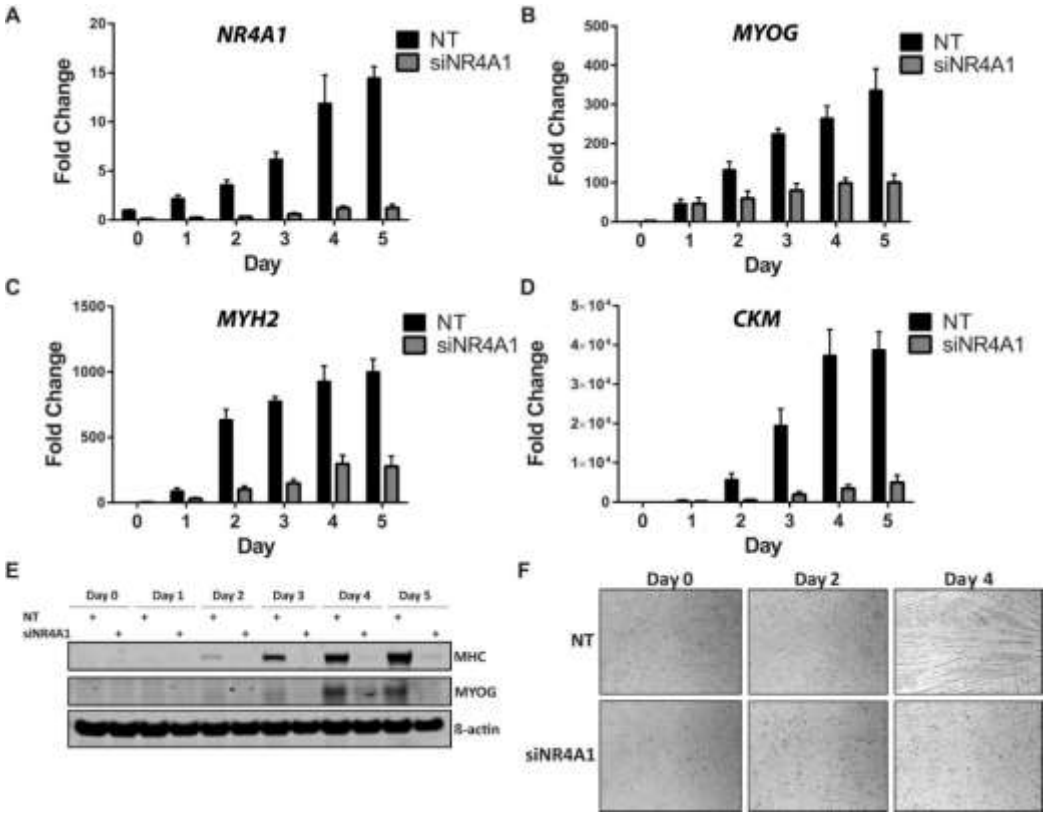

Figure 4-4. Knockdown of *NR4A1* delays differentiation in LHCN.
(A-D) LHCN were transfected with 10 nm non-targeting control (NT) and siRNA for *NR4A1* (siNR4A1) prior to differentiation for 5 days with cells collected every day beginning on Day 0. The mRNA levels of *NR4A1* and differentiation markers were measure and normalized to NT Day 0, which was set to 1. Data shown is the average of 3 independent experiments. **(E)** Protein levels for MHC, MYOG, and β-actin were detected in a western blot. **(F)** Bright-field images with 10X magnification were taken on Days 0, 2, and 4 with representative images shown.

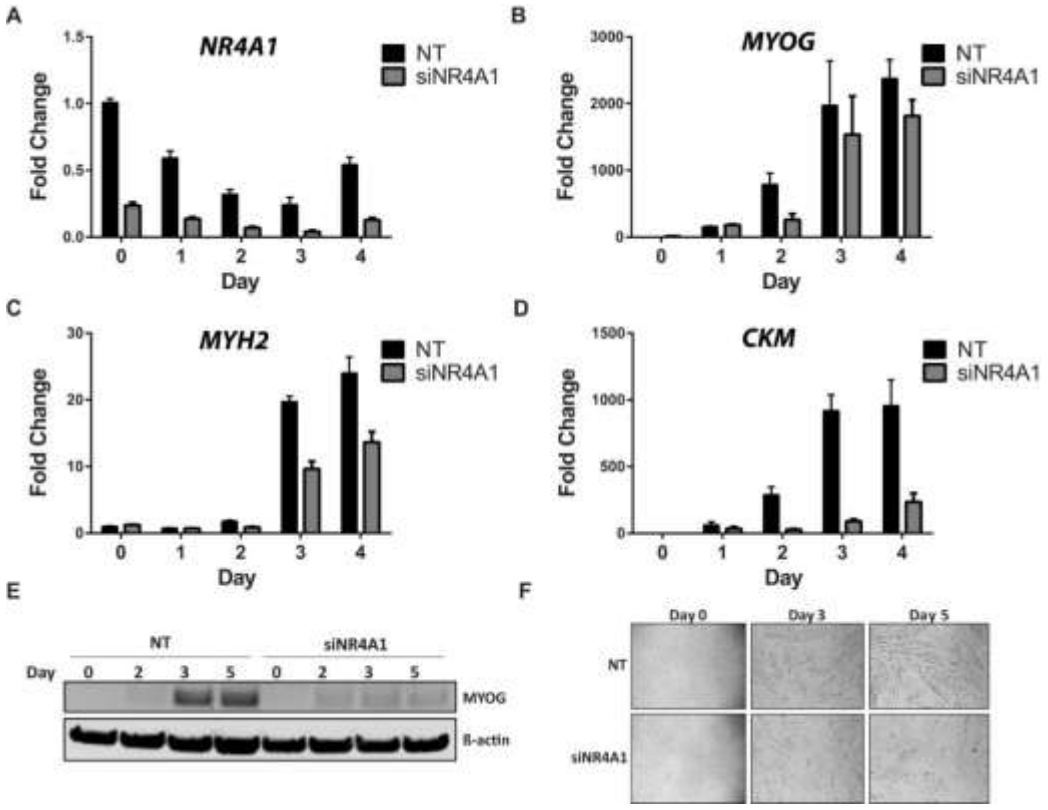

Figure 4-5. ***NR4A1*** **knockdown delays differentiation in SkMC.**
(A-D) SkMC were transfected with 10 nm NT and siNR4A1 prior to differentiation for 5 days. The mRNA levels of *NR4A1* and differentiation markers were measured and normalized to NT Day 0, which was set to 1. Data shown is the average of 3 independent experiments. **(E)** Protein levels of MYOG and β-actin were measured in a western blot. **(F)** Bright-field images at 10X magnification were taken on Days 0, 3, and 5 with representative images shown.

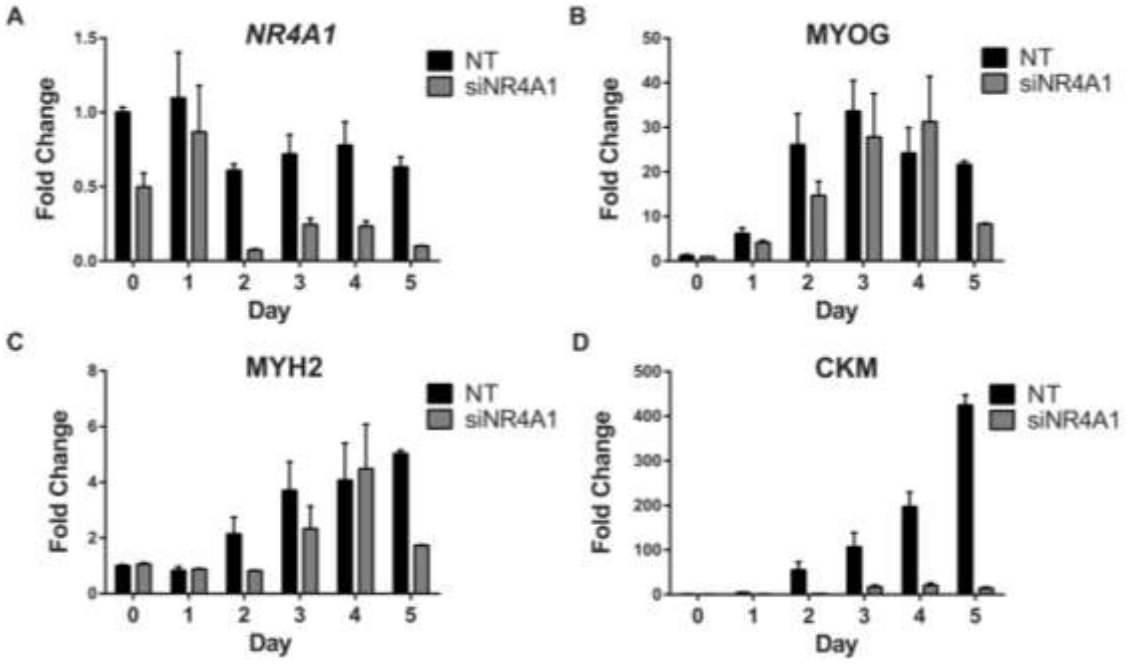

Figure 4-6. ***NR4A1*** **knockdown delays differentiation in HSMM.**
(A-D) HSMM were transfected with 10 nm NT and siNR4A1 prior to differentiation for 5 days. The mRNA levels of *NR4A1* and differentiation markers were measured and normalized to NT Day 0, which was set to 1. Data shown is the average of 2 independent experiments.

(**Figure 4-7C-D**). Interestingly, knockdown of *ZNF148* induced differentiation in the presence of growth media, demonstrating the strong repressive power of endogenous ZNF148 in the control cells (**Figure 4-7A**). Accordingly, *NR4A1* expression was also induced more than 15 fold in the cells with siZNF148 under growth conditions and more than 10 fold under differentiation conditions (**Figure 4-7B**).

Conclusions

In all, we discovered a novel role for NR4A1 in skeletal muscle differentiation. *NR4A1* expression increases during the myogenic program in LHCN, and knockdown of *NR4A1* results in decreased expression of differentiation markers in LHCN, SkMC, and HSMM human skeletal muscle cells. This supports a previous study showing that *Nr4a1* increases during the differentiation of mouse C2C12 skeletal muscle cells [142, 476]. However in that study, they noted that differentiation still occurred in the absence of *Nr4a1*. Perhaps the difference in our results depends on the species and cell type as those studies were conducted in murine cells whereas these studies were performed in human cells.

Although *NR4A1* was increased in LHCN, it was not increased in SkMC or HSMM. This could be an artifact of the immortalized state of LHCN, whereas SkMC and HSMM are primary cells. We would expect NR4A1 to have similar expression patterns in LHCN and HSMM since these two cell types both originate from adult tissue, whereas SkMCs are isolated from gestational tissue. Another difference in cell type is that SkMCs are precursors and therefore less differentiated, whereas HSMM and LHCN are myoblasts committed to becoming muscle. Even though *NR4A1* was not increased in SkMC and HSMM, knockdown of *NR4A1* in these cells still resulted in decreased expression of myogenic markers. Therefore it would appear that NR4A1 is important during myogenesis. It was also interesting to observe the higher induction of *NR4A1* in cells transfected with NT. Perhaps there are some off target effects of the NT that inadvertently increase *NR4A1* levels. It would be reasonable to test additional NT controls to determine if these effects are specific to the original NT used here. Furthermore, we found that *NR4A2* was decreased during the differentiation of LHCN and SkMC while *NR4A3* was increased in all 3 cell types. This suggests that *NR4A2* overexpression should hypothetically inhibit differentiation while *NR4A3* overexpression should promote it. Perhaps the NR4A family has synergistic effects where myogenesis is enhanced upon *NR4A2* knockdown and *NR4A1* and *NR4A2* overexpression.

Since there are several stages of differentiation, it is of interest to determine the stage in which NR4A1 is acting. Perhaps it is promoting the initial stages of cell cycle arrest, or maybe it is mainly functioning during a later stage. One way to elucidate this would be to determine the differentiation and fusion indices. The differentiation index is a measure of the number of nuclei in myosin-positive cells which indicates how differentiated the cells are whereas the fusion index is a measure of the number of nuclei in each myotube, indicating the ability of myoblasts to fuse into myotubes [477]. Calculating these indices would help determine the effect of NR4A1 on how well the

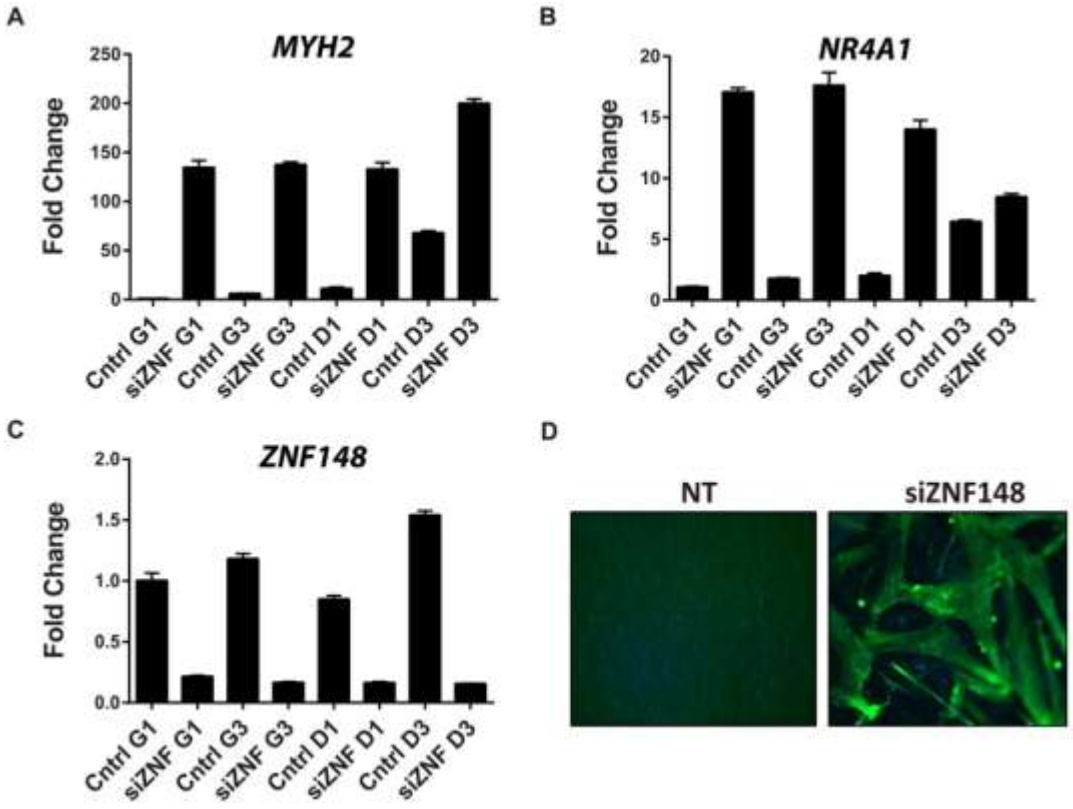

Figure 4-7. Knockdown of *ZNF148* rapidly induces differentiation in LHCN. **(A-C)** LHCN cells were transfected with 10 nM Cntrl or siZNF and cultured in either growth (G1, G3) or differentiation (D1, D3) media with cells collected on Days 1 and Day 3. The resulting mRNA levels of *MYH2*, *ZNF148*, and *NR4A1* were measured and normalized to Cntrl G1, which was set to 1. **(D)** Immunofluorescence was performed to detect MHC (green) and DAPI (blue) in cells with *ZNF148* knocked down. Images shown are 20X magnification and were taken 2 days after the addition of differentiation media.

cells are able to differentiate and fuse. It is possible that knocking down *NR4A1* is blocking or delaying myoblast fusion without effecting differentiation.

On the other hand, NR4A1 may actually be more involved in the metabolism aspect. Upon knockdown of *NR4A1* in LHCN, SkMC, and HSMM, the muscle marker *CKM* is dramatically downregulated whereas the other muscle markers *MYOG* and *MYH2* are only modestly decreased. Many studies have shown that CKM is an essential enzyme during energy homeostasis and mediates the synthesis of creatine phosphate (CrP) and ADP by transferring the gamma phosphate from ATP. During intense exercise when there is a high demand for energy, CKM will then synthesis ATP from CrP and ADP [478]. As expected, decreased CKM contributes to the gradual loss of muscle mass and function during aging *in vivo* [478, 479]. As mentioned, NR4A1 has been shown to promote glucose and oxidative metabolism in murine skeletal muscle [148, 474]. Therefore, it is quite possible that the role of NR4A1 in muscle is primarily related to metabolism. To confirm this, expression of additional genes involved in metabolism should be analyzed upon knockdown or overexpression of NR4A1 during the differentiation of LHCN, SkMC, and HSMM cells.

Interestingly, knockdown of *ZNF148* resulted in elevated levels of *NR4A1* as well as rapid formation of myotubes, even in the absence of differentiation growth factors. This most likely occurs by the direct repression of *NR4A1* by ZNF148 at the promoter of *NR4A1* as previously shown in pancreatic β-cells. This demonstrates a possible mechanism by which ZNF148 and NR4A1 may be controlling skeletal muscle differentiation. These results also highlight ZNF148 as a therapeutic target in diseases such as RMS where the cells have failed to differentiate.

CHAPTER 5. DISCUSSION*

Regulation of *NR4A1* by miRNAs in Cancer

The regulation of *NR4A1* by miRNAs was previously unknown, and the function of NR4A1 in pediatric cancers is currently undetermined. In this study, we found that miR-124 directly targets *NR4A1* and that NR4A1 is upregulated in multiple pediatric cancer cell lines, including rhabdomyosarcoma, neuroblastoma, and medulloblastoma cell lines. We focused on elucidating the function of NR4A1 and miR-124 in medulloblastoma cells, and showed that exogenous expression of miR-124 in Daoy medulloblastoma cells decreased the cells' proliferation and viability.

Several previous reports suggest that miR-124 might also regulate *NR4A1* indirectly. In pancreatic beta-cells, Sp1 binds to the promoter of *NR4A1* and increases NR4A1 levels [480]. Interestingly, during neuronal differentiation of mesenchymal stem cells, miR-124 targets Sp1 mRNA and decreases Sp1 expression [481]. The results of these studies suggest that miR-124 may indirectly decrease NR4A1 expression by decreasing Sp1. In addition, NR4A1 binds to the promoter of several target genes, including *E2F1*, *CCND2* (cyclin D2), *BIRC5* (survivin), *TXNDC5*, *CDK4*, and *STAT5A* [231, 233, 438, 439]. Consistent with these reports, we showed that overexpression of miR-124 decreased expression of these 6 target genes. By promoting expression of these genes, NR4A1 exerts its effects on cell proliferation and survival. Aberrant overexpression of NR4A1 can, therefore, lead to tumor growth and cancer progression. **Figure 5-1** summarizes our discovery of the miR-124/NR4A1 functional relationship in the context of relevant previous reports.

Medulloblastoma is a highly malignant primary brain tumor that originates in the cerebellum. It is also the most common malignant brain tumor in children, with patients having a 50%-80% chance of survival depending on the specific tumor type and other factors [482, 483]. There are 4 subgroups of medulloblastoma: Wnt, Shh, Group 3, and Group 4 [484]. A study profiling miRNAs in Shh MB tumors found 30 miRNAs that were downregulated in tumors with high Gli1, one of which was miR-124 [485]. Another miRNA profiling study also found that miR-124 in a Shh MB mouse model was downregulated compared to that in 1-month-old cerebella [486]. Furthermore, a study profiling 19 human medulloblastomas found that miR-124 was downregulated in the Wnt- and Shh-associated MBs [487]. Additionally, one study of miRNA profiles in 34 human primary medulloblastomas found that miR-124 was downregulated (subtypes were not specified) [488].

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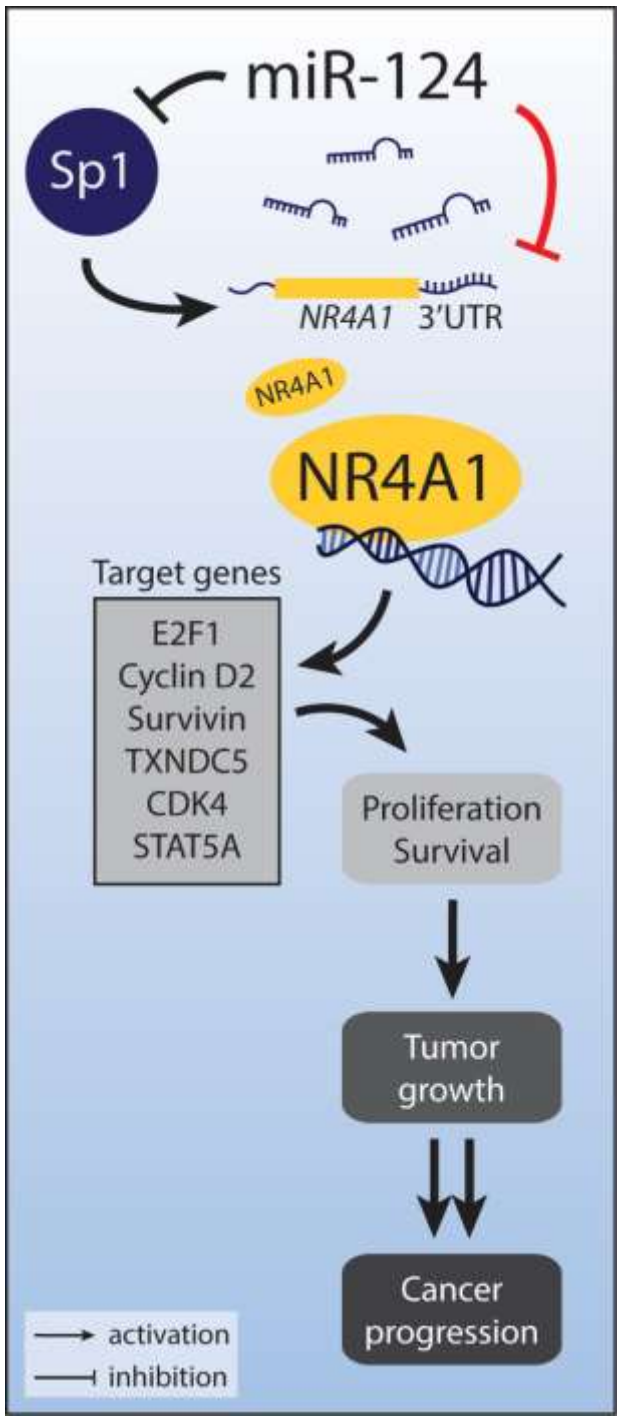

Figure 5-1. Overview of NR4A1 regulation by miR-124.
NR4A1 can be directly targeted by miR-124, as revealed by our studies reported here (indicated by the red line), or indirectly affected by miR-124 via Sp1. NR4A1 may act through several downstream target genes to promote cell proliferation and survival.

Interestingly, miR-124 is the most abundant miRNA in the brain [464] and functions to promote neuronal differentiation [489], regulate neural stem cells [490], and induce differentiation in glioma stem cells [491]. Pierson et al. first showed that miR-124 targets CDK6 in medulloblastoma cell lines and that miR-124 is downregulated in medulloblastoma cells lines and tumors [465]. Silber et al. went on to show that miR-124 inhibits proliferation of medulloblastoma cells via cell-cycle arrest during G1 and that these results are more dramatic in cells with higher CDK6 levels. Importantly, inducible overexpression of miR-124 *in vivo* significantly reduced tumor growth generated by subcutaneous injection of D425 medulloblastoma cells [467]. Li et al. also found downregulation of miR-124 in 29 medulloblastomas and showed that miR-124 targets SLC16A1, which functions to efflux lactic acid during aerobic glycolysis. The authors suggest that inhibition of SLC16A1 by miR-124 decreases intracellular pH to a lethal level, leading to the observed growth inhibition in medulloblastoma cell lines upon overexpression of miR-124 [466]. It is clear that miR-124 has an important tumor-suppressive role in medulloblastoma and that it acts through various target genes. Our research provides, for the first time, an additional target gene of miR-124, *NR4A1*, which has known oncogenic roles in adult solid tumors.

As summarized in **Figure 5-1**, miR-124 may also indirectly downregulate *NR4A1* by directly targeting the mRNA of Sp1, resulting in reduced Sp1 and NR4A1 levels. Additionally, miR-124 is predicted to target *CCND2* and *TXNDC5*, which are both target genes of NR4A1. Furthermore, miR-124 is predicted to target *RXRA* (RXRα) and *GSK3B* (GSK3β). RXRα and NR4A1 heterodimerize and either translocate to the mitochondria to induce apoptosis or bind to the promoters of NR4A1 target genes to modulate transcription [162, 175]. GSK3β suppresses NR4A1 activity by phosphorylating NR4A1 in colorectal cancer [418]. Therefore, it is possible for miR-124 to exert both positive and negative effects directly and indirectly on NR4A1, depending on the specific cellular context.

The main type of neuron that makes up the cerebellum is the granule neuron. We found an inverse expression pattern whereby *NR4A1* is upregulated and miR-124 is downregulated in Daoy medulloblastoma cells and in undifferentiated murine GNPs. Similar to Daoy medulloblastoma cells, in undifferentiated GNPs, the level of *NR4A1* is high and that of miR-124 is low. Once the GNPs differentiate into mature granule neurons, *NR4A1* levels drop dramatically and miR-124 expression increases. These observations are consistent with those in studies showing that miR-124 promotes neuronal differentiation [489-491] and that miR-124 levels in 1-month-old mouse cerebellar tissue are higher than those in P6 GNPs [486]. The dramatic decrease in *NR4A1* expression after differentiation suggests that the levels of *NR4A1* need to be reduced before the cells can develop into mature neurons.

It is reasonable to hypothesize that increased levels of miR-124 are needed to decrease *NR4A1* for differentiation to occur. Aberrant downregulation of miR-124 might block differentiation and promote tumorigenesis, warranting the future investigation of the regulation of miR-124 levels. The miR-124 promoter has been reported to be hypermethylated in pancreatic cancer [492], hepatocellular carcinoma [493, 494],

ulcerative colitis, [495] and acute lymphoblastic leukemia [496]. It is therefore of interest to analyze the promoter of miR-124 before and after differentiation to identify any changes in methylation status and to identify proteins that may bind to the promoter of miR-124, thereby affecting endogenous levels of miR-124.

NR4A1 can reportedly enhance neuronal outgrowth and differentiation: both dibutyryl-cAMP (dbcAMP) and trichostatin A (TSA) promote neurite outgrowth in PC12 rat pheochromocytoma cells by inducing *Nr4a1* expression via acetylated Lys14 of histone H3, and knockdown of *Nr4a1* inhibits dbcAMP and TSA-induced neurite outgrowth [497, 498]. NR4A1 overexpression also promotes neurite formation in PC12 cells [498]. However, the opposite phenotype is observed in a murine macrophage cell line. Oxidized low-density lipoprotein (oxLDL) induces mature macrophages to differentiate into dendritic cells and induces NR4A1 expression in vascular cells. However, when NR4A1 is overexpressed in RAW264.7 murine macrophages, differentiation into dendritic cells is inhibited in the presence of oxLDL [499]. This comes back to the key point that NR4A1 functions are heavily dependent on cellular context, so it is possible for NR4A1 to have opposing functions in different tissues and cell types.

Daoy cells are classified as desmoplastic cerebellar medulloblastoma [500]; however, researchers have found that this cell line does not mimic any of the 4 subtypes of medulloblastoma. It would be useful to compare the expression level of NR4A1 in the brain to that in medulloblastoma tumors, but unfortunately there are not substantial published data showing NR4A1 levels in human medulloblastoma. However, two databases show low basal expression of NR4A1 in healthy cerebellum. The Brain Transcriptome Database shows that in situ hybridization images of the cerebellum have very little NR4A1 signal [501]. In addition, the Genotype-Tissue Expression project found that expression levels of NR4A1 in different parts of the brain, including the cerebellum, were much lower than those in other normal tissue types [502]. Given our finding of elevated *NR4A1* in Daoy cells, NR4A1 may have an oncogenic role in medulloblastoma, which is supported by our data showing that exogenous overexpression of NR4A1 promotes Daoy cell viability and proliferation and that *NR4A1* knockdown results in the opposite phenotype.

Upon overexpression of miR-124 in Daoy cells, NR4A1 mRNA and protein levels and the mRNA levels of NR4A1 target genes decreased, showing that miR-124 affects not only NR4A1 expression but also the transcriptional activity of NR4A1. Stable overexpression of miR-124 led to decreases in cell viability, cell proliferation, and microtumor spheroid size, suggesting therapeutic potential for miR-124 in treating cancer.

Role of NR4A1 in Skeletal Muscle Differentiation

NR4A1 appears to play a variety of roles in muscle, including the enhancement of glucose and oxidative metabolism in C2C12 mouse myoblasts and *in vivo* in mouse and

rat muscle [148, 474]. In addition, older studies have shown that *Nr4a1* expression increases during C2C12 differentiation [142]. These cells still retained the potential to differentiate when *Nr4a1* was knocked down, however several genes involved in lipolysis were decreased. This led to the conclusion that NR4A1 is important for energy expenditure and has potential therapeutic value in treating obesity [142]. More recently, NR4A1 was found to promote myofiber size and muscle mass *in vivo* through activation of the Akt-mTOR-S6K cascade [475]. After global and muscle-specific knockout of *Nr4a1*, the muscle mass was decreased. Importantly, primary myoblasts from these mice formed fewer and smaller myotubes [475]. The above studies were all performed using either animal models or murine C2C12 cells. We wanted to determine the function of NR4A1 during skeletal muscle differentiation using human cell lines.

During the differentiation of LHCN, we found that *NR4A1* expression increases by about 4 fold. These results are consistent with previous studies showing a 3-5 fold increase of *Nr4a1* in C2C12 cells [142, 476]. It is of interest to determine which stage NR4A1 is becoming expressed. Since *NR4A1* expression does not increase until Day 3 of differentiation, it would appear that its presence is not crucial for the initiation of differentiation. However when *NR4A1* was knocked down via siRNA, the induction of differentiation was severely delayed alongside decreased expression of muscle markers in LHCN, SkMC, and HSMM. It is also possible that the fusion of myoblasts is being blocked or delayed. Thus we will need to determine exactly which stage NR4A1 is playing a role. Nonetheless, this suggests that NR4A1 is important during the differentiation of skeletal muscle cells.

Previous studies in our lab have demonstrated that the zinc finger transcription factor ZNF148 (ZBP-89) acts as a suppressor of the myogenic program as deduced from knockdown studies showing rapid formation of myotubes in LHCN cells when *ZNF148* was expressed at extremely low levels. As a well-known repressor of transcription, ZNF148 is likely acting by binding to the promoters of genes involved in myogenesis and inhibiting their expression. A recent study links ZNF148 to NR4A1 by demonstrating the ability of ZNF148 to bind the promoter of *NR4A1* and block its expression in pancreatic β-cells [480]. This discovery led us to question whether ZNF148 is transcriptionally regulating *NR4A1* during skeletal muscle differentiation. Upon knockdown of *ZNF148* via siRNA in LHCN cells, *NR4A1* expression was rapidly induced by 15 fold along with high levels of *MHC*. This suggests that ZNF148 represses *NR4A1* in skeletal muscle cells and that downregulation of *ZNF148* by siRNA relieves this repression. To confirm this, we will need to perform ChIP and probe for ZNF148 at the promoter of *NR4A1* in LHCN cells.

Furthermore, it is unclear whether *NR4A1* induction is due to the knockdown of *ZNF148* or as a result of induced differentiation. To elucidate this, we knocked down both *ZNF148* and *NR4A1* and tested whether siZNF148 was still able to induce differentiation. If ZNF148 functions through NR4A1, we would expect abnormal differentiation in the absence of NR4A1. However, we found that differentiation was still induced at the same rate (data not shown). Therefore, it would appear that the mechanism of siZNF148-mediated differentiation is not solely through NR4A1. Since ZNF148 is a

transcription factor and is known to target and suppress numerous genes, it may still be acting through NR4A1 in addition to many other genes. Therefore, knockdown of *NR4A1* alone would not be sufficient to rescue this phenotype. Another member of the NR4A family, NR4A3, may be compensating for the lack of NR4A1. Our study among other studies has shown that *NR4A3* increases during skeletal muscle differentiation and therefore it may have redundant roles with NR4A1 [503]. This would not be out of the ordinary as other studies have shown overlapping roles for NR4A1 and NR4A3 in other tissues. It is of interest to determine if *NR4A3* expression increases upon *NR4A1* knockdown. Perhaps NR4A3 partially rescues the effects of *NR4A1* knockdown and therefore differentiation still occurs, albeit at a slower rate. In addition, a double knockdown of both *NR4A1* and *NR4A3* may have a more profound effect on differentiation by completely preventing the initiation of differentiation. A recent study found that knockdown of *NR4A3* in HSMM blocked the formation of myotubes, suggesting that NR4A3 is required for skeletal muscle differentiation [504].

The regulation and function of NR4A1 during differentiation is of interest. Although NR4A1 does not appear to be a mechanism for ZNF148, that does not mean that ZNF148 is not a mechanism for NR4A1. ZNF148 may need to be suppressed during differentiation in order for *NR4A1* expression to increase. We found that *ZNF148* mRNA levels are unchanged during differentiation (data not shown), however its activity may still be suppressed. A proteomics study performed in our lab shows that ZNF148 protein levels decrease by almost 25% during LHCN differentiation (data not shown). Reduced ZNF148 activity would result in the inability to transcriptionally inhibit *NR4A1*, which would result in increased *NR4A1* expression. The mechanism by which NR4A1 potentially enhances differentiation is likely through transcriptional activation of genes important for the initiation and progression of the myogenic program as depicted in **Figure 5-2**. NR4A1 has been shown to induce expression of CKM, although it apparently does not bind the promoter [476]. Furthermore, 685 genes were recently identified by ChIP-seq as being directly regulated by NR4A1 in AML cells [505]. Interestingly, NR4A1 was found to transcriptionally activate ENO3 [505], a gene that our lab identified in the proteomics study to increase by 26 fold during muscle differentiation. ENO3 has been previously shown to increase during differentiation and has roles during muscle development [506]. Furthermore, ENO3 was found to be regulated by ETS transcription factors, which were recently discovered to interact with NR4A1 to transcriptionally activate NR4A1 target genes [505]. Therefore, it is quite possible that NR4A1 enhances myogenesis by promoting the expression of genes involved in this process.

It may appear difficult to find a connection between the two main projects discussed here. The proliferative role of NR4A1 in cancer seems contradictory to its pro-myogenic role in skeletal muscle. The key to these differential functions may depend on the expression levels of NR4A1. For instance in cancer, *NR4A1* appears to be highly elevated. This substantial upregulation may be necessary for the proliferative effects of NR4A1. Conversely, *NR4A1* expression increases during skeletal muscle differentiation (only in LHCN), although not to the same degree as in cancer. During normal muscle differentiation, NR4A1 may be acting as a co-driver to ensure the smooth and timely

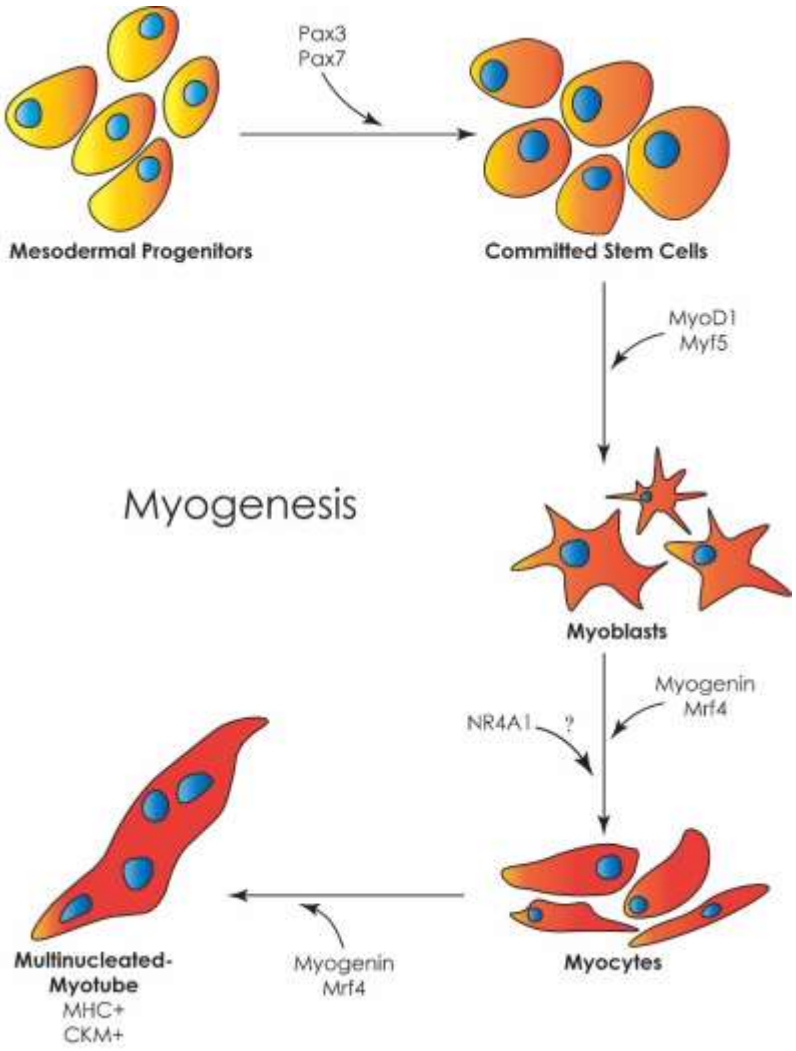

Figure 5-2. Overview of the myogenic program.
Muscle differentiation begins with mesodermal progenitors that express Pax3 and Pax7. Transcriptional activity by Myf5 allows the cells to either progress into brown adipose or into myoblasts after the addition of MyoD activity. Other transcription factors such as myogenin and possibly NR4A1 allow the cells to become myocytes. These cells fuse into multinucleated myotubes that will make up the muscle fibers.

differentiation of skeletal muscle cells, most likely by transcriptionally activating genes involved in myogenesis.

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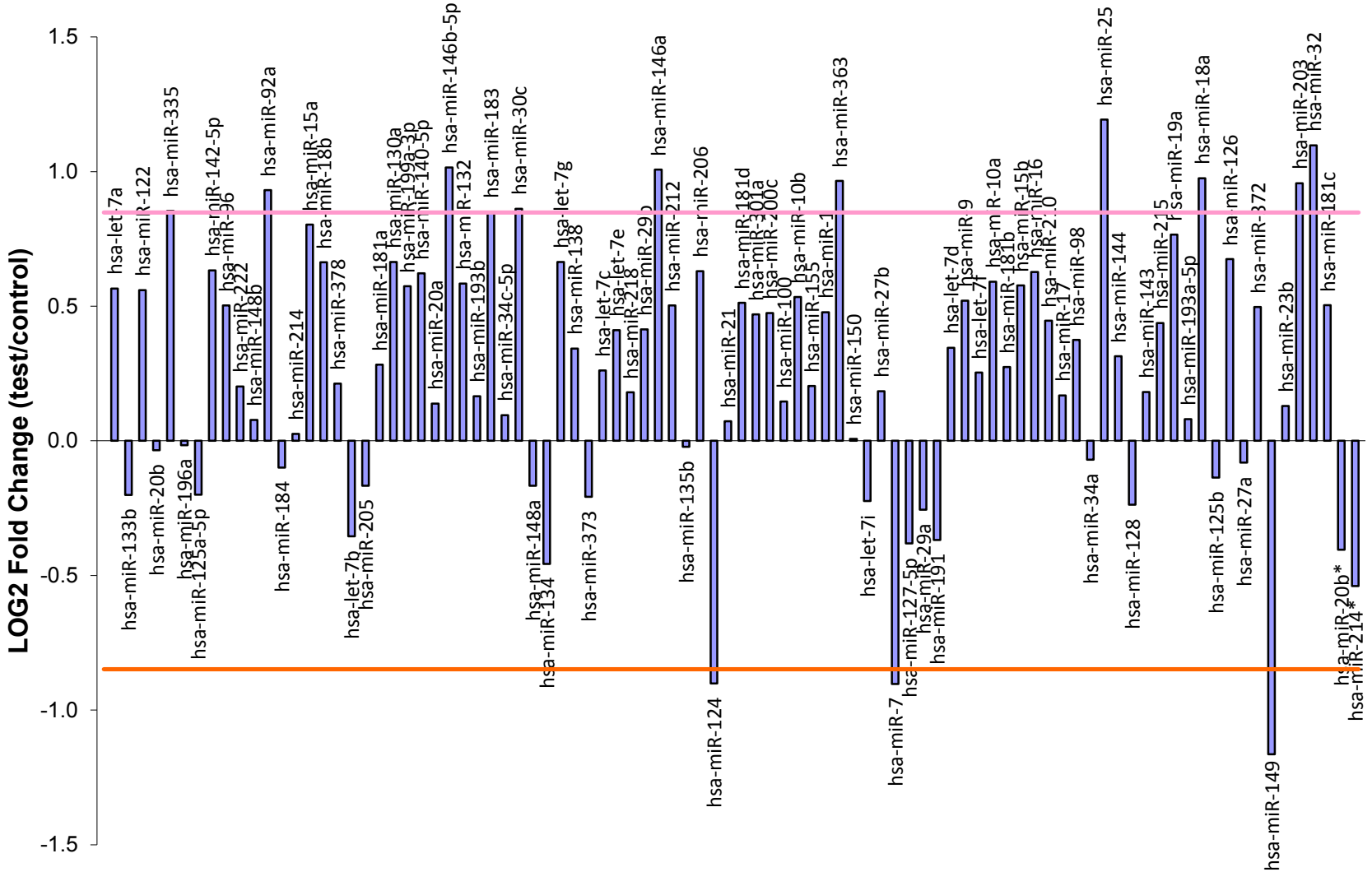
APPENDIX. SUPPLEMENTAL DATA FOR CHAPTER 3

Figure A-1. miR-124 decreases *NR4A1* expression in miRNA array.
The Cancer miRNAs Transcriptome PCR Array containing cDNA from HeLa cells transfected with one of the 90 cancer-related miRNAs, as described in Materials and Methods, was used to detect *NR4A1* expression and identify miRNAs that target *NR4A1*. The resulting gene expression of *NR4A1* is displayed as Log2 with horizontal lines indicating the cutoff value (as suggested by the manufacturer) at which *NR4A1* gene expression is considered to be significant. Three miRNAs, including miR-124, were found to decrease *NR4A1* expression.


LOG2 Fold Change (test/control)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
hsa-let-7a
hsa-miR-133b
hsa-miR-122
hsa-miR-20b
hsa-miR-335
hsa-miR-196a
hsa-miR-125a-5p
hsa-miR-142-5p
hsa-miR-96
hsa-miR-222
hsa-miR-148b
hsa-miR-92a
hsa-miR-184
hsa-miR-214
hsa-miR-15a
hsa-miR-18b
hsa-miR-378
hsa-let-7b
hsa-miR-205
hsa-miR-181a
hsa-miR-130a
hsa-miR-199a-3p
hsa-miR-140-5p
hsa-miR-20a
hsa-miR-146b-5p
hsa-miR-132
hsa-miR-193b
hsa-miR-183
hsa-miR-34c-5p
hsa-miR-30c
hsa-miR-148a
hsa-miR-134
hsa-let-7g
hsa-miR-138
hsa-miR-373
hsa-let-7c
hsa-let-7e
hsa-miR-218
hsa-miR-29
hsa-miR-146a
hsa-miR-212
hsa-miR-135b
hsa-miR-206
hsa-miR-124
hsa-miR-21
hsa-miR-181d
hsa-miR-301a
hsa-miR-200c
hsa-miR-100
hsa-miR-10b
hsa-miR-155
hsa-miR-363
hsa-miR-150
hsa-let-7i
hsa-miR-27b
hsa-miR-7
hsa-miR-127-5p
hsa-miR-29a
hsa-miR-191
hsa-let-7d
hsa-miR-9
hsa-let-7f
hsa-miR-10a
hsa-miR-181b
hsa-miR-15b
hsa-miR-16
hsa-miR-17
hsa-miR-98
hsa-miR-34a
hsa-miR-25
hsa-miR-144
hsa-miR-128
hsa-miR-143
hsa-miR-215
hsa-miR-19a
hsa-miR-193a-5p
hsa-miR-18a
hsa-miR-125b
hsa-miR-126
hsa-miR-27a
hsa-miR-372
hsa-miR-149
hsa-miR-23b
hsa-miR-203
hsa-miR-32
hsa-miR-181c
hsa-miR-20b*
hsa-miR-214*

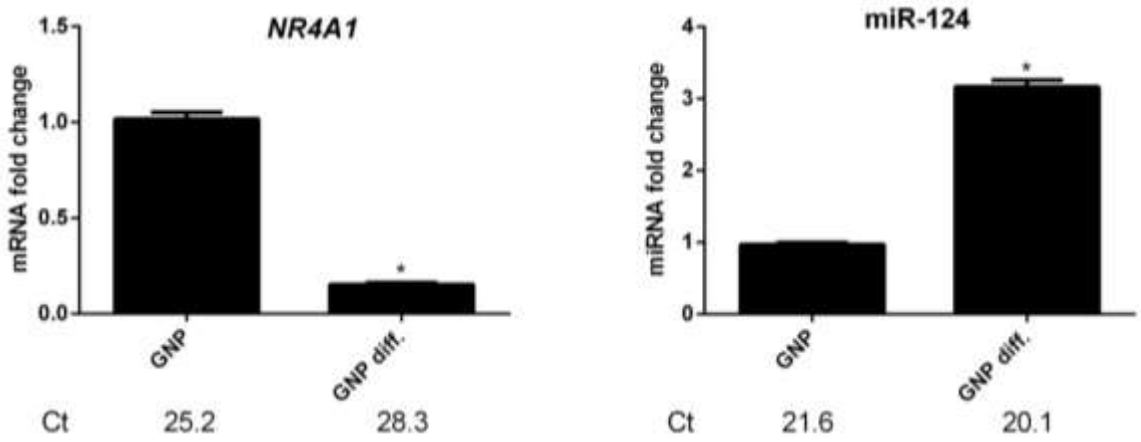


Figure A-2. NR4A1 and miR-124 have inverse expression in granule neurons. *NR4A1* and miR-124 expression were measured in granule neuron precursors (GNPs) harvested from P7 mice. The GNPs were cultured for 24 hours, allowing enough time for the cells to differentiate (GNP diff.) before being collected for expression analysis. The fold change for the GNPs was set to 1. The internal control for *NR4A1* was *GAPDH* and the control for miR-124 was snoRNA 202. The data shown are the average of 3 independent experiments with the average Ct values indicated below each graph. * indicates $p < 0.0001$.

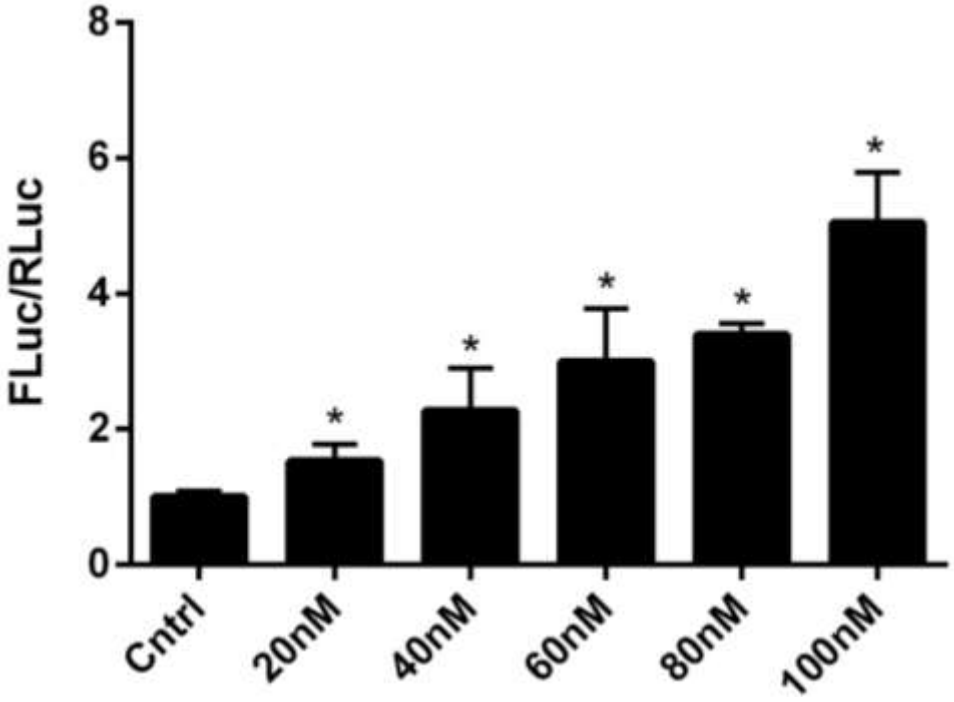


Figure A-3. An inhibitor of miR-124 increases NR4A1 activity.
Daoy cells were transfected with the NR4A1-3′UTR reporter plasmid (NR4A1-3′UTR-Luc) and either the Exiqon miR-124 inhibitor at the indicated concentrations or the control molecule (Cntrl), resulting in increased luciferase activity as the concentration of the inhibitor increased. Data shown are representative of 2 independent experiments. * indicates $p < 0.05$.

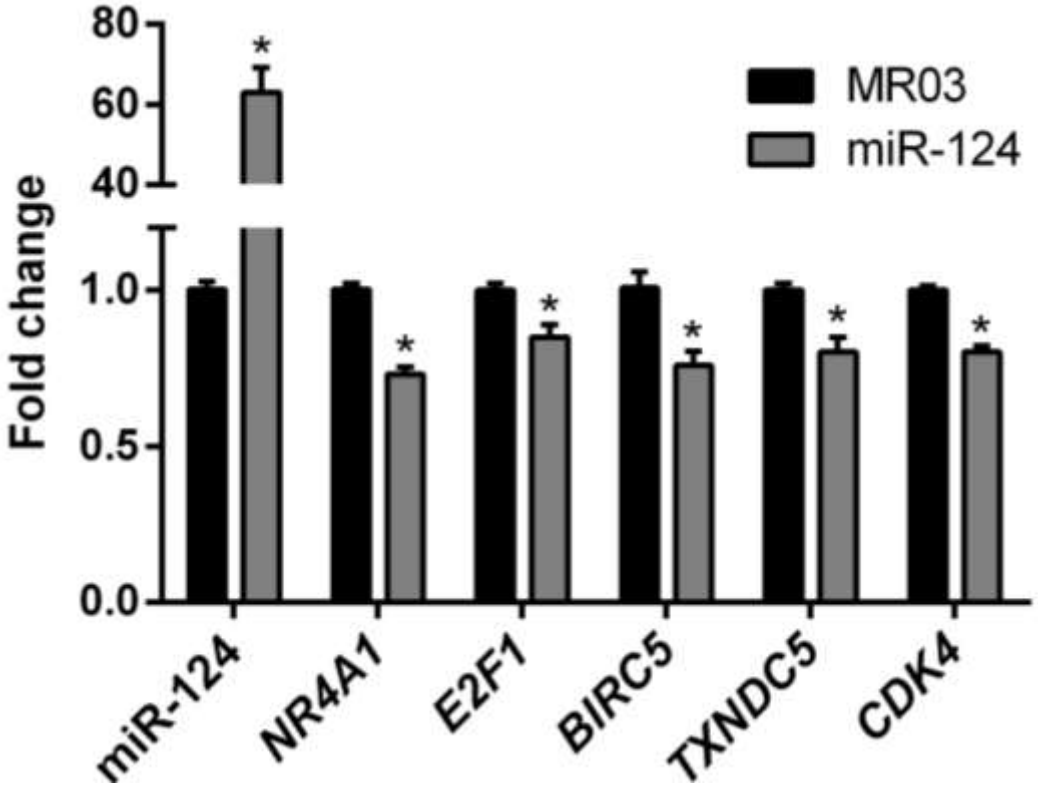


Figure A-4. miR-124 decreases levels of NR4A1 target genes in 293T cells. Transfection of 293T cells with miR-124 decreased the levels of *NR4A1* and its target genes, *E2F1*, *BIRC5* (survivin), *TXNDC5*, and *CDK4,* compared to those of cells transfected with the vector control (MR03). The data shown are the average of 3 independent experiments. * indicates $p < 0.01$.

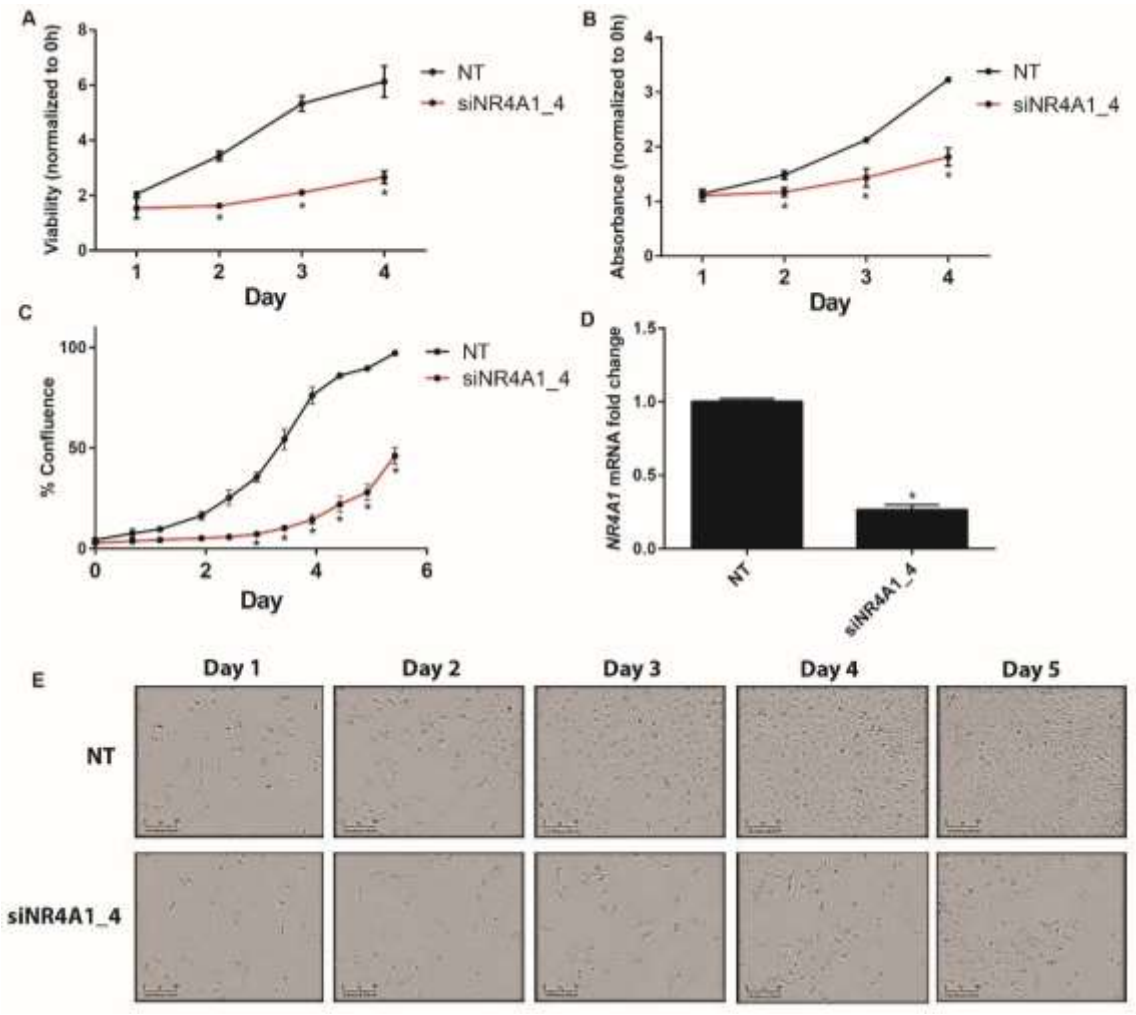


Figure A-5. ***NR4A1*** **knockdown decreases cell viability and proliferation.** **(A)** Daoy cells were transfected with 20 nM of the individual siNR4A1_4 or non-targeting control (NT), and cell viability was measured via the CellTiter-Glo assay every day for 4 days. Viability for each day was normalized to that of Day 0 (0 hours), and statistical significance was calculated for each day; $*p < 0.0001$. **(B)** Cells were stained with crystal violet every day for 4 days to measure proliferation over time. The absorbance was measured and normalized to that of Day 0 (0 hours). The statistical significance was calculated for each day; $*p < 0.01$. **(C)** Proliferation was monitored via the IncuCyte live-cell imager. Cell confluence was averaged, with 4 replicates of each condition; $*p < 0.0001$. **(D)** *NR4A1* mRNA was significantly ($p < 0.0001$) decreased after transfecting Daoy cells with siNR4A1_4. **(E)** Images shown for each NT and siNR4A1_4 panel over 5 days are the same image view within the same well and are representative of 3 independent experiments with 4 wells for each condition. These images correspond to the data in C. Data shown in D are the average of 4 independent experiments. Data shown in A and B are representative of 3 independent experiments, and data in C and E are representative of 2 independent experiments.

VITA

Alexa Farmer was born in Norfolk, VA in 1988 and grew up in Fairfax, VA. She graduated from Virginia Tech in 2011 with a Bachelor of Science in Biological Systems Engineering. She then started the Integrated Biomedical Sciences Program at the University of Tennessee Health Science Center. In May of 2012, she joined the laboratory of Dr. Taosheng Chen at St. Jude Children's Research Hospital to study the function and regulation of the nuclear receptor NR4A1 by miRNAs in cancer. She graduated in August of 2016 with the degree of Doctor of Philosophy with a concentration in cancer and developmental biology. She will pursue a postdoctoral fellowship in Tucson, AZ.